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I, CASSANDRA RICHARDS, ACTING TEAM LEADER EXAMINATION SUPPORT & SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PQ 3049 for a patent by COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION and AUSTRALIAN WOOL RESEARCH AND PROMOTION ORGANISATION filed on 24 September 1999.

WITNESS my hand this
Thirty-first day of October 2000

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COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH OGANISATION

AND

AUSTRALIAN WOOL RESEARCH AND PROMOTION ORGANISATION

A U S T R A L I A

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"Method of improving plant productivity "

The invention is described in the following statement:

METHOD OF MODIFYING PLANT PRODUCTIVITY

FIELD OF THE INVENTION

The present invention relates generally to a method of modifying plant productivity comprising expressing in a plant cell, tissue or organ one or more genes capable of facilitating a plant's ability to utilise soil phosphorus. More particularly, the present invention provides a method of increasing plant productivity comprising expressing in the root of a plant an isolated nucleic acid molecule encoding a phytase polypeptide for a time and under conditions sufficient for said phytase to be secreted from the root, and preferably, further comprising modifying the chemistry of the soil around the root using an organic acid. The present invention extends to novel phytase-encoding genes; genetic constructs which are useful for performing the inventive method; and to transgenic plants produced therewith having improved productivity compared to their otherwise isogenic counterparts.

GENERAL

Those skilled in the art will be aware that the invention described herein is subject to variations and modifications other than those specifically described. It is to be understood that the invention described herein includes all such variations and modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Throughout this specification, unless the context requires otherwise the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

This specification contains nucleotide and amino acid sequence information (referenced herein by the prefix "SEQ ID NO:<400>"), prepared using the programme PatentIn Version 2.0. The Sequence Listing is presented herein after the bibliography. Each nucleotide or amino acid sequence is identified in the Sequence Listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, etc). The length, type of sequence (DNA, protein (PRT), etc) and source organism for each nucleotide or amino acid sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide and amino acid sequences referred to in the specification are defined by the information provided in numeric indicator field <400> followed by the sequence identifier (eg. <400>1, <400>2, etc).

The designation of nucleotide residues referred to herein are those recommended by the IUPAC-IUB Biochemical Nomenclature Commission, wherein A represents Adenine, C represents Cytosine, G represents Guanine, T represents thymine, Y represents a pyrimidine residue, R represents a purine residue, M represents Adenine or Cytosine, K represents Guanine or Thymine, S represents Guanine or Cytosine, W represents Adenine or Thymine, H represents a nucleotide other than Guanine, B represents a nucleotide other than Adenine, V represents a nucleotide other than Thymine, D represents a nucleotide other than Cytosine and N represents any nucleotide residue.

Amino acid designations referred to herein are listed in Table 1.

TABLE 1

Amino Acid	Three-letter Abbreviation	One-letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Any amino acid as above	Xaa	X

BACKGROUND TO THE INVENTION

In light of the dwindling supply of land available for agriculture, intensive agriculture production is an imperative for the purposes of feeding the increasing worldwide population. To achieve this end, it is necessary to increase the productivity of agriculture plants. High productivity is of great agricultural and horticultural value,

because increased growth reduce times-to-harvest and yield of crop plants. This improvement is of considerable value in the case of forage crops.

It is well known that phosphorus may boost or even optimise plant productivity. Soil phosphorus may originate from the deposition of organic material in the soil, which form can account for at least 50-85% of total soil phosphorus. However, organic forms of soil phosphorus, such as, for example, inositol phosphorus (soil phytate), may also account for a substantial component total soil phosphorus. For example, in Australian soils, phytate accounts for up to 38% of total organic phosphorus, and organic phosphorus may account for 50-85% of total soil phosphorus.

Present methods for boosting plant productivity include the application of phosphate-based fertilisers to the soil. High costs of intensive agriculture, particularly in respect of producing agronomically-important crops, are incurred by the requirement to apply phosphate-based fertilisers to the soil. This is especially evident in regions where the soils are deficient in forms of phosphorus that are readily utilisable by plants. Additionally, there is a considerable environmental cost associated with the use of phosphate fertilisers in particular, due to run-off entering the water catchment and resulting in algal blooms under appropriate conditions.

In spite of the benefits to be derived from providing phosphorus to plants in terms of increased productivity, the use of phosphate-based fertilisers has declined recently, in part due to the high economic costs associated therewith and in part due to the high environmental costs. This decline in phosphate-based fertiliser usage has occurred in regions where soils are deficient in forms of phosphate that are available to plants. This has meant a reduction in plant productivity, particularly in those regions having phosphorus-deficient soils.

Notwithstanding the high proportion of total phosphorus present in the soil in the form of soil phytate, plants almost exclusively derive their phosphorus requirement from soluble phosphate anions, and possess a very limited capacity to directly obtain

phosphorus from soil phytate, because phytate is not absorbed by plant roots and further, because phytate is inefficiently hydrolysed to inositol and phosphorus in the soil. Microorganisms and fungi in the soil are known to contain phytase enzymes that catalyse the conversion of phytate to inositol and inorganic phosphate and phytase-encoding genes of *Aspergillus niger* have been described previously in United States Patent No. 5, 436, 156 issued on 25 July, 1995 (hereinafter "Van Gorcom *et al.*, 1995"). Additionally, Van Ooijen *et al.* (United States Patent No. 5, 593, 963 issued on 14 January, 1997) expressed the *Aspergillus ssp.* phytase gene in the cells of plants, in particular the seeds, with a view to increasing the level of available phosphorus in feedstock containing the transgenic plants. However, the transgenic plants produced by Van Ooijen *et al.* do not possess improved phosphorus nutrition by virtue of an ability to *utilise* soil phytate.

In work leading up to the present invention, the present inventors sought to improve the phosphorus nutrition and yield of plants without the extensive application of phosphate-based fertilisers, by increasing or improving the ability of a plant to utilise phytate, in particular soil phytate. Surprisingly, the present inventors have found that by ectopically expressing phytase enzyme in the roots, and secreting the phytase into the extracellular environment outside the root, the ability of the plant to utilise phytate as a source of phosphorus is markedly improved. Plants produced according to the inventive method provide considerably benefits to the agriculture sector, in the form of reduced economic and environmental costs, and improved plant productivity relative to their otherwise isogenic counterparts. These benefits are further enhanced if the inventive method is coupled with the step of modifying the chemistry of the soil around the root using an organic acid.

SUMMARY OF THE INVENTION

One aspect of the invention provides a method of improving the phosphorus nutrition of a plant comprising ectopically expressing in the root of a plant an isolated nucleic acid molecule encoding a phytase polypeptide for a time and under conditions sufficient for said phytase to be secreted from the root.

In a preferred embodiment of the invention, the phytase-encoding nucleic acid molecule is secreted from root cells in the region of the root tip and/or the zone of elongation, that divide more rapidly than those cells that are more distal to the root tip.

Preferably, the secretion of phytase from the root provides a high local concentration of active phytase enzyme in the vicinity surrounding those root cells that are involved in active phosphorus uptake. In work leading up to the present invention the inventors found that mere cell damage or sloughing that occurs during the movement of the root through the soil fails to provide sufficient phytase activity in the region surrounding those root cells involved in phosphorus uptake, and that an active secretion mechanism is important to achieve the improved phosphorus nutrition of the invention, Accordingly, a preferred embodiment of the present invention provides for the phytase enzyme to be produced as a fusion polypeptide with a secretory signal sequence that is active in plant cells and capable of achieving protein transport not merely outside of a root cell, but outside the root surface. In a particularly preferred embodiment, the phytase enzyme is produced as a fusion polypeptide with the leader sequence of the carrot extensin polypeptide to facilitate extracellular targeting of phytase outside the root surface.

In an alternative embodiment, the present invention provides a method of improving the phosphorus nutrition of a plant comprising:

(i) ectopically expressing in the root of a plant an isolated nucleic acid molecule encoding a phytase polypeptide for a time and under conditions sufficient for said phytase to be secreted from the root; and

(ii) modifying the chemistry of the soil around the root or other growth medium around the root using an organic acid for a time and under conditions sufficient to solubilise phosphorus produced by the action of said phytase enzyme on phytate.

The application of the inventive method results in the production of plants having higher biomass production than otherwise isogenic counterparts, including higher rates

of hypocotyl and epicotyl production, leading to a greater accumulation of biomass and larger plants, without the need for extensive application of phosphate-based fertilisers in soils that comprise phytate or to which phytate has accumulated as a result of past agricultural practice. By virtue of its relative insolubility compared to phosphate anions, and the fact that phytate is less readily absorbed than phosphate anions, phytate provides environmental advantages relative to super-phosphates. Accordingly, the present invention provides for the production and use of hitherto unknown "phytate-based fertilisers" in conjunction with the inventive method.

Accordingly, a further aspect of the invention contemplates a plant fertiliser comprising phytate and/or a fertiliser composition comprising phytate and a suitable carrier for application to plants and/or the soil.

The present invention further extends to the plants produced by the performance of the inventive method.

A further aspect of the present invention provides an isolated nucleic acid molecule encoding a phytase polypeptide and having at least about 92% nucleotide sequence identity to SEQ ID NO: <400> 1 or SEQ ID NO: <400> 9 and/or which is capable of hybridising to SEQ ID NO: <400> 1 or SEQ ID NO: <400> 9 or a complementary nucleotide sequence thereto under high stringency hybridisation conditions. Preferably, the isolated nucleic acid molecule of the invention is derived from a microbial source such as, for example, the filamentous fungi *Aspergillus ssp.*

Alternatively, the isolated nucleic acid molecule comprises a nucleotide sequence that encodes an amino acid sequence having at least about 95% identity to the sequence set forth in SEQ ID NO: <400> 2 or SEQ ID NO: <400> 10 or an enzymically-active fragment thereof.

In one embodiment, the isolated nucleic acid molecule encoding phytase is obtainable by the method of:

a) hybridising under at least low stringency conditions plant genomic DNA, RNA or cDNA derived therefrom with one or more nucleic acid probes or primers of at least 10 nucleotides in length for a period of time and under conditions sufficient to form a double-stranded nucleic acid molecule, wherein said probes or primers comprise a nucleotide sequence obtainable from SEQ ID NO: <400> 1 or SEQ ID NO: <400> 9 or a nucleotide sequence that is complementary thereto;

b) detecting the hybridised nucleic acid molecule; and

c) isolating said hybridised nucleic acid molecule comprising said genetic sequence.

In a particularly preferred embodiment, the isolated nucleic acid molecule of the invention comprises the nucleotide sequence set forth in SEQ ID NO: <400> 1 or SEQ ID NO: <400> 9 or a fragment thereof encoding an active phytase enzyme. Alternatively, the the isolated nucleic acid molecule comprises a nucleotide sequence that encodes the amino acid sequence set forth in SEQ ID NO: <400> 2 or SEQ ID NO: <400> 10 or an enzymically-active fragment thereof.

A further aspect of the present invention extends to gene constructs comprising a phytase-encoding nucleotide sequence connected in-frame to a secretory signal-encoding nucleotide sequence, and placed operably in connection with a promoter sequence that is operable in the root cells of a plant. Preferably, the phytase-encoding nucleotide sequence comprises the *Aspergillus niger 9-1* nucleotide sequence set forth in SEQ ID NO: <400> 1 or SEQ ID NO: <400> 9 or a homologue or derivative thereof as described herein. Preferably, the secretory-signal-encoding nucleotide sequence is the carrot extensin secretory signal or equivalent.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a copy of a representation of a nucleotide sequence alignment between the open reading frames of the *Aspergillus niger PhyA* gene (*phyA.seq;* GenBank Accession No. M94550) and the partial *A. niger 9-1* gene (9-1.seq) obtained by the

present inventors, produced using the CLUSTAL W algorithm of Thompson *et al* (1994). Numbering refers to the nucleotide positions in the *PhyA* gene sequence.

Figure 2 is a copy of a representation of an amino acid sequence alignment between the *Aspergillus niger* PhyA polypeptide (*phyA.pro;* GenBank Accession No. M94550) and the partial *A. niger 9-1* polypeptide (9-1.pro) obtained by the present inventors, produced using the CLUSTAL W algorithm of Thompson *et al* (1994). Numbering refers to the amino acid positions in the *PhyA* polypeptide.

Figure 3 is a copy of a representation of the pPLEX vector plasmid designated pBS389. This plasmid contains the sub-clover stunt virus (SCSV) region 1 promoter (Sc1 Pr; International Patent Application No. PCT/AU95/00552) and SCSV region 3 terminator (Sc3 3′; International Patent Application No. PCT/AU95/00552) operably connected to a kanamycin-resistance gene (nptII) for expression in plants, flanked by the *Agrobacterium tumefaciens* left-border (LB) and right-border (RB) integration sequences; a bacterial-operable spectinomycin/streptomycin resistance gene (Sp-R/St-R); an *Agrobacterium* origin of replication (oriVRK2) and *E.coli* origin of replication (oricolE1) and intergenic spacer (IS1/oriT). Positions of restriction sites are indicated.

Figure 4 is a copy of a representation of the plasmid pART 7. Plasmid pART7 is a vector containing bacterial two origins of replication (f1 ori and ori), an ampicillin resistance gene for bacterial selection (AmpR). Plasmid pART7 also contains two *NotI* restriction sites flanking a CaMV 35S promoter-multiple cloning site(MCS)-NOS 3′ cassette, wherein the MCS permits cloning of structural genes in operable connection with said promoter and terminator sequences.

Figure 5 is a copy of a representation of the plasmid pAER02, containing the carrot extensin leader-encoding sequence (ext) in-frame with the *A. niger phyA* gene (phyA) and placed operably in connection with the CaMV 35S promoter sequence and OCS terminator sequence. This vector is based upon plasmid pBS389 (Figure 3).

Figure 6 is a copy of a representation of the plasmid pAER04, containing the carrot extensin leader-encoding sequence (ext) in-frame with the *A. niger 9-1* gene (9-1) and placed operably in-connection with the CaMV 35S promoter sequence and OCS terminator sequence. This vector is based upon plasmid pBS389 (Figure 3).

Figure 7 is a copy of a photographic representation showing the growth of *Arabidopsis thaliana* plants on soluble phosphate-containing media [PO_4^{2-}] (left panels), phytate (middle panels), or without phosphorus (right panels). Plants shown in the top row were transformed with a control plasmid pBS389. Plants shown in the bottom row were transformed with a gene construct comprising the CaMV 35S promoter driving expression of the chimeric extensin::*phyA* structural gene upstream of the *ocs terminator*, and these plants express an extensin-phytase fusion polypeptide. Significantly higher productivity was observed for plants expressing the extensin-phytase fusion polypeptide compared to control plants, on media containing phytate as a source of phosphorus.

Figure 8 is a copy of a photographic representation showing the growth of transgenic *Arabidopsis thaliana* plants on phytate. Plants shown in the left panel were transformed with a plasmid vector comprising the phytase gene under control of the CaMV 35S promoter and express phytase without a leader sequence to facilitate secretion. Plants shown in the right panel were transformed with the extensin/phytase gene construct comprising the CaMV 35S promoter driving expression of the chimeric extensin:: *9-1* structural gene placed upstream of the *ocs* terminator, and these plants express an extensin-phytase fusion polypeptide. Significantly higher productivity was observed for plants expressing the extensin-phytase fusion polypeptide compared to plants expressing phytase but unable to target the phytase to the extracellular space.

Figure 9 is a copy of a graphical representation showing the amount of phytase-labile phosphorus (filled-in bars) as a proportion of total organic phosphorus that have been extracted from soils using HCl, water or citric acid. Data show higher levels of soluble organic phosphorus and a greater proportion of phytase-labile phosphorus in citric acid extracts.

Figure 10 is a copy of a representation of a nucleotide sequence alignment between the open reading frames of the *Aspergillus niger PhyA* gene (*phyA.seq;* GenBank Accession No. M94550) and the complete *A. niger 9-1* gene (9-1.seq) obtained by the present inventors (SEQ ID NO: <400> 9), produced using the CLUSTAL W algorithm of Thompson *et al* (1994). Numbering refers to the nucleotide positions in the *PhyA* gene sequence.

Figure 11 is a copy of a representation of an amino acid sequence alignment between the *Aspergillus niger* PhyA polypeptide (*phyA.pro;* GenBank Accession No. M94550) and the complete *A. niger 9-1* polypeptide (9-1.pro) obtained by the present inventors (SEQ ID NO: <400> 10), produced using the CLUSTAL W algorithm of Thompson *et al* (1994). Numbering refers to the amino acid positions in the *PhyA* polypeptide.

Figure 12 is a copy of a photographic representation showing the growth of *Arabidopsis thaliana* plants after 40 days on soluble phosphate-containing media [PO_4^{2-}] (right panel) or without phosphorus (left panel). Plants were transformed with pBS389 lacking a phytase-encoding sequence. Significantly higher productivity was observed for plants grown on phosphorus-contianing media, as expected.

Figure 13 is a copy of a photographic representation showing the growth of transgenic *Arabidopsis thaliana* plants after 40 days on phytate-contianing media. Plants shown in the right panel were transformed with a plasmid vector comprising the phytase gene under control of the CaMV 35S promoter and express phytase as a fusion with carrot extensin secretory signal sequence to facilitate secretion. Plants shown in the left panel were transformed with the phytase gene (i.e. expressed phytase without the extensin signal peptide) under control of the CaMV promoter. Significantly higher productivity on phytate was observed for plants expressing the extensin-phytase fusion polypeptide compared to plants expressing phytase but unable to target the phytase to the extracellular space.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

One aspect of the invention provides a method of improving the phosphorus nutrition of a plant comprising ectopically expressing in the root of a plant an isolated nucleic acid molecule encoding a phytase polypeptide for a time and under conditions sufficient for said phytase to be secreted from the root.

As used herein, the term "phosphorus nutrition" shall be taken to refer to the utilisation by a plant of an external source of phosphorus in any form, including organic phosphorus and/or phosphate anion and/or phytate and/or phosphate and/or orthophosphate and/or pyrophosphate, amongst others. By "external source of phosphorus" is meant phosphorus that is taken up by the plant from the external environment.

An "improved phosphorus nutrition" refers to a greater ability of the plant to utilise an existing phosphorus source in the soil or growth medium in which said plant grows.

Accordingly, the present invention is directed to a method of improving the ability of a plant to utilise an external source of phosphorus. In a particularly preferred embodiment, the external source of phosphorus is phytate.

The term "phytate" shall be taken to refer to any storage phosphorus source comprising inositol phosphate, including aggregates and polymers thereof, and phytin, a generic term applied to complex salts of phytic acid (for a review *see* Graf, 1986).

The term "phytase polypeptide" refers to any amino acid sequence, peptide, oligopeptide, polypeptide, or protein molecule, with or without additional non-amino acid substituents or non-naturally-occurring amino acid substituents, that is capable of catalysing the removing a phosphorus-containing moiety from phytate as hereinbefore defined. Preferably, a phytase polypeptide will further be capable of catalysing the conversion of phytate to inositol and phosphorus, which may be in any form, such as, for example, an anion, or metal complex, a transition metal complex, or a weak acid, amongst others. Those skilled in the art will be aware of those forms

of soil phosphate that are readily utilisable by plants, and the present invention clearly extends to phytase enzymes capable of converting phytate to any such form of soil phosphate.

Whilst the present invention is not limited by the source of phytase, the phytase polypeptide of the invention is preferably derived from a plant, microorganism, or animal cell. Those skilled in the art will be aware that phytases are widely-occurring enzymes in nature, derivable from bacteria, such as, for example, *Bacillus subtilis* (Paver and Jagannathan, 1982), *Pseudomonas* (Cosgrove, 1970); yeasts, such as, for example, *Saccharomyces cerevisiae* (Nayini and Markakis, 1984); fungi, such, for example, *Aspergillus fumigatus* (Wyss *et al.*, 1999), Aspergillus *terreus* (Yamada *et al.*, 1986), *A. niger* (Mullaney *et al.*, 1991; van Hartingsveldt *et al.*, 1993); plants (Loewus, 1990) such as, for example, maize (Laboure *et al.*, 1993; Hubel and Beck, 1996; Maugenest *et al.*, 1997), potato (Gellady and Lefebvre, 1990), and soybean (Gibson *et al.*, 1988), amongst others. Conveniently, the phytase enzyme employed in the performance of the present invention possesses high specific activity and/or high Vmax in a soil environment and/or low Km for phytate in a soil environment.

In a particularly preferred embodiment, the phytase enzyme employed in the performance of the present invention is derived from a fungus, more preferably *Aspergillus ssp.*, and even more preferably from *A. niger.* As exemplified herein, the *A. niger* phytase enzymes comprising the amino acid sequences set forth in SEQ ID NO: <400> 2 and/or SEQ ID NO: 10 and/or SEQ ID NO: <400> 4 provide high biomass production when expressed in the roots of transgenic plants and secreted therefrom into the surrounding growth medium.

For the purposes of nomenclature, the amino acid sequence set forth in SEQ ID NO: <400> 2 relates to the partial *A. niger* phytase polypeptide 9-1, which has been produced by expression of the *A. niger 9-1* gene (SEQ ID NO: <400> 1), a variant of the *A. niger phyA* gene that is suitable for expression in plants. The internal region of the nucleotide and amino acid sequences is missing from these partial sequences. For convenience, the sequences of the ends of the nucleic acid molecule and

polypeptide are presented as single molecules, the end-points of which can be determined by reference to the alignments presented in Figures 1 and 2, and by reference to the complete sequences provided in SEQ ID NOs: <400> 9 and <400> 10.

The amino acid sequence set forth in SEQ ID NO: <400> 10 relates to the complete *A. niger* phytase polypeptide 9-1, which has been produced by expression of the *A. niger 9-1* gene (full-length sequence presented as SEQ ID NO: <400> 10), a variant of the *A. niger phyA* gene that is suitable for expression in plants.

The amino acid sequence set forth in SEQ ID NO: <400> 4 relates to a variant of the *A. niger* PhyA polypeptide (Mullaney *et al.*, 1991; Van Hartingsveldt *et al.*, 1993; GenBank Accession No. M94550), having the leader sequence removed and a different translation start site inserted relative to the naturally-occurring PhyA polypeptide. To express the PhyA polypeptide in plants, the present inventors modified the corresponding *phyA* gene sequence to remove the endogenous *A. niger* leader sequence-encoding nucleotide sequence and intron sequence, and introduce a new translation start site immediately prior to and in-frame with, the nucleotide sequence encoding the mature PhyA polypeptide.

As used herein, the terms "in-frame" and "in the same reading frame" refer to one or more codons of a nucleotide sequence being in the same open reading frame as one or more other codons of said nucleotide sequence. Similarly, the term "in-frame fusion" refers to the linkage between two or more heterologous nucleotide sequences such that the amino acid sequences encoded therefor are expressed in the same reading frame and, as a consequence, as a single polypeptide molecule.

The phytase polypeptide may be expressed throughout the length of the root, or alternatively, in a localised region of the root, preferably in the zone of elongation and/or the root tip. Conveniently, expression occurs in the epidermal cells and/or the cortex, to facilitate the secretion of the phytase to the root surface, either by reducing the number of cell layers through which the phytase must be transported or

alternatively, by facilitating transport of the phytase to the epidermal cells.

Preferably, secretion of the phytase polypeptide from the root cells in which it is expressed is achieved by expressing the phytase as an in-frame fusion polypeptide with a secretory signal sequence capable of directing transport of the phytase to the root surface. According to this embodiment, the secretory signal sequence may be placed at the N-terminal and/or C-terminal end of the phytase polypeptide. Those skilled in the art will be aware that such signal sequences have been demonstrated to be operable in either configuration. Alternatively, or in addition, a secretory signal sequence may be embedded in the phytase polypeptide, the only requirement being that the embedding of the secretory signal sequence in the phytase does not inactivate the phytase enzymic activity in the soil or growth medium. The present invention further encompasses the use of a cryptic secretory signal sequence, either as an in-frame fusion with phytase or alternatively, by mutation of a region of the phytase polypeptide to produce an amino acid variant phytase polypeptide, such as a substitutional variant or insertional variant or deletional variant, that comprises a cryptic secretory signal sequence therein.

Substitutional variants are those in which at least one residue in the phytase amino acid sequence has been removed and a different residue inserted in its place. Amino acid substitutions are typically of single residues, but may be clustered depending upon functional constraints placed upon the polypeptide; insertions will usually be of the order of about 1-10 amino acid residues. and deletions will range from about 1-20 residues. Preferably, amino acid substitutions will comprise conservative amino acid substitutions, such as those described *supra*.

Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the phytase protein. Insertions can comprise amino– terminal and/or carboxyl terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Generally, insertions within the amino acid sequence will be smaller than amino or carboxyl terminal fusions, of the order of about 1 to 4 residues.

Deletional variants are characterised by the removal of one or more amino acids from the phytase sequence.

Phytase amino acid variants may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis and the like, or by recombinant DNA manipulations. The manipulation of DNA sequences to produce variant proteins which manifest as substitutional, insertional or deletional variants are well known in the art. For example, techniques for making substitution mutations at predetermined sites in DNA having known sequence are well known to those skilled in the art, such as by M13 mutagenesis or other site-directed mutagenesis protocol.

Preferred secretory signal sequences according to this embodiment of the invention are derived from plants, fungi, yeasts, bacteria or animal cells, the only requirement being that they function in the root of a plant. Such function can be readily determined without undue experimentation, by determining the level of phytase transported to the cell surface or root surface following its ectopic expression in the root as an in-frame fusion with the secretory signal sequence. Alternatively, or in addition, the efficacy of the signal sequence can be tested by determining the ability of a plant ectopically expressing the phytase as an in-frame fusion with the signal sequence to grow on phytate as a source of phosphorus. As exemplified herein, and as shown in Figure 9, plants that ectopically express phytase as an in-frame fusion with the secretory signal sequence derived from the carrot extensin protein, exhibit improved growth on phytate relative to otherwise isogenic non-transformed plants. Using such tests, those skilled in the art can readily determine the optimum secretory signal sequence for performing the present invention, and the optimum placement of said secretory signal sequence relative to the phytase polypeptide.

The secretory signal sequence is conveniently derived from the full-length potato patatin polypeptide (Iturriaga *et al*,1989; Li *et al.*, 1997), the tobacco PR-S polypeptide (Comelissen *et al.* 1986; Pen *et al.*, 1993), the lupin acid phosphatase (LASAP 1; Wasaki *et al.*, 1999) polypeptide or the carrot extensin polypeptide (Chen and Varner, 1985).

In a particularly preferred embodiment, the secretory signal sequence is derived from the carrot extensin polypeptide and placed at the N-terminus of the phytase polypeptide.

Nucleotide sequences of the secretory signal-encoding nucleotide sequences of the carrot extensin and lupin acid phosphatase genes are set forth herein, as SEQ ID NOs: <400> 5 and <400> 7, respectively. The amino acid sequences of the carrot extensin and lupin acid phosphatase secretory signal peptides are also set forth herein, as SEQ ID NOs: <400> 5 and <400> 7, respectively.

The word "express" or variations such as "expressing" and "expression" as used herein shall be taken in their broadest context to refer to the transcription of a particular genetic sequence to produce sense or antisense mRNA or the translation of a sense mRNA molecule to produce a peptide, polypeptide, oligopeptide, protein or enzyme molecule. In the case of expression comprising the production of a sense mRNA transcript, the word "express" or variations such as "expressing" and "expression" may also be construed to indicate the combination of transcription and translation processes, with or without subsequent post-translational events which modify the biological activity, cellular or sub-cellular localization, turnover or steady-state level of the peptide, polypeptide, oligopeptide, protein or enzyme molecule.

Without being bound by any theory or mode of action, the expression of phytase in the root cell and its subsequent secretion to the root surface provides a localised high concentration of active phytase enzyme the is capable of diffusing into the soil to catalyse the conversion of phytate into inositol and phosphorus, such that the phosphorus is then able to be absorbed or actively taken up by the root.

The term "ectopic expression" refers to the *de novo* and/or increased expression of a peptide, oligopeptide, polypeptide or protein from an introduced nucleic acid molecule, such as, for example, by means of transfection or transformation of a cell, tissue or organ with nucleic acid encoding the peptide, oligopeptide, polypeptide or protein. Ectopic expression can also be achieved by the infection or transformation of a cell,

tissue or organ with a foreign organism containing nucleic acid encoding the peptide, oligopeptide, polypeptide or protein.

Accordingly, to ectopically-express a phytase polypeptide comprising an in-frame fusion with a secretory signal sequence, it is necessary to produce a corresponding nucleic acid molecule encoding both the secretory signal sequence and the phytase polypeptide in the same reading frame. This may be achieved by those skilled in the art without undue experimentation.

For the ectopic expression of a peptide, oligopeptide, polypeptide or protein in a plant cell, tissue or organ, the nucleic acid molecule encoding said peptide, oligopeptide, polypeptide or protein in a plant-expressible format, such as, for example, in an appropriate gene construct comprising a promoter sequence operably connected to said nucleic acid molecule, and optionally a transcription termination sequence comprising a polyadenylation signal, amongst others.

By "expressible format" is meant that the isolated nucleic acid molecule is in a form suitable for being transcribed into mRNA and/or translated to produce a protein, either constitutively or following induction by an intracellular or extracellular signal, such as an environmental stimulus or stress (anoxia, hypoxia, temperature, salt, light, dehydration, etc) or a chemical compound such as an antibiotic (tetracycline, ampicillin, rifampicin, kanamycin) hormone (eg. gibberellin, auxin, cytokinin, glucocorticoid, etc), hormone analogue (iodoacetic acid (IAA), 2,4-D, etc) , metal (zinc, copper, iron, etc), or dexamethasone, amongst others. As will be known to those skilled in the art, expression of a functional protein may also require one or more post-translational modifications, such as glycosylation, phosphorylation, dephosphorylation, or one or more protein-protein interactions, amongst others. All such processes are included within the scope of the term "expressible format".

the term "plant-expressible format" refers to an expressible format that pertains to expression of proteins in plant cells, tissues or organs.

Preferably, the ectopic expression of phytase is effected by introducing an isolated nucleic acid molecule encoding phytase, such as a cDNA molecule, genomic gene, synthetic oligonucleotide molecule, mRNA molecule or open reading frame, to a plant cell, tissue or organ, operably in connection with a promoter sequence that is capable of conferring expression in a plant root cell, albeit not necessarily exclusively in the root cell.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences derived from a classical eukaryotic genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or are capable of conferring expression on a structural gene sequence (i.e. the protein-coding region of a gene) in a tissue-specific manner, conveniently in the roots.

In the resent context, the term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of a nucleic acid molecule in a plant cell, tissue or organ.

Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression and/or to alter the spatial expression and/or temporal expression of a nucleic acid molecule to which it is operably connected. For example, copper-responsive, glucocorticoid-responsive or dexamethasone-responsive regulatory elements may be placed adjacent to a heterologous promoter sequence driving expression of a nucleic acid molecule to confer copper inducible, glucocorticoid-inducible, or dexamethasone-inducible expression respectively, on said nucleic acid molecule.

Included within the scope of the present invention is the use of strong constitutive promoter sequences, cell-specific promoter sequences, inducible promoter sequence, tissue-specific promoter sequences, organ-specific promoter sequences, and

constitutive promoter sequences that have been modified to confer expression in a particular part of the plant at any one time, such as by integration of said constitutive promoter within a transposable genetic element (*Ac, Ds, Spm, En*, or other transposon).

The term "constitutive" will be known by those skilled in the art to indicate that expression is observed predominantly throughout the plant, albeit not necessarily in every cell, tissue or organ under all conditions. In the present context, a preferred strong constitutive promoter is one which confers a high level of ectopic expression on a phytase structural gene to which it is operably connected, predominantly throughout the plant and at least in the root, albeit not necessarily in every cell, tissue or organ under all conditions.

The term "cell-specific" shall be taken to indicate that expression is predominantly in a particular plant cell or plant cell-type, albeit not necessarily exclusively in that plant cell or plant cell-type. In the present context, a preferred cell-specific promoter will confer expression on a phytase gene in at least one cell type of the root, preferably, a root epidermal cell or root cortical cell.

Similarly, the term "tissue-specific" shall be taken to indicate that expression is predominantly in a particular plant tissue or plant tissue-type, albeit not necessarily exclusively in that plant tissue or plant tissue-type. In the present context, a preferred tissue-specific promoter will confer expression on one or more tissues of the root, such as, for example, in the zone of elongation, the root vasculature, or the root tip.

Similarly, the term "organ-specific" shall be taken to indicate that expression is predominantly in a particular plant organ albeit not necessarily exclusively in that plant organ. In the present context, a preferred organ-specific promoter will confer expression on a phytase gene throughout the root.

Those skilled in the art will be aware that an "inducible promoter" is a promoter the transcriptional activity of which is increased or induced in response to a developmental,

chemical or physical stimulus.

Preferably, the promoter is a root-specific, phosphate-regulated promoter derived from a phosphate transport gene, such as, for example, the phosphate transport genes of *Arabidopsis thaliana* or barley plants, which are induced under conditions of phosphate deficiency in the plant. These promoters are obtainable from CSIRO Tropical Agriculture, Queensland, Australia.

The present invention is not to be limited by the choice of promoter sequence and those skilled in the art will readily be capable of selecting appropriate promoter sequences for use in regulating appropriate expression of phytase or modified phytase from publicly-available or readily-available sources, without undue experimentation.

Placing a phytase-encoding nucleic acid molecule under the regulatory control of a promoter sequence, or in operable connection with a promoter sequence, means positioning said nucleic acid molecule such that expression is controlled by the promoter sequence.

A promoter is usually, but not necessarily, positioned upstream, or at the 5′-end, and within 2 kb of the start site of transcription, of the nucleic acid molecule which it regulates.

In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting (i.e., the gene from which the promoter is derived). As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting (i.e., the gene from which it is derived). Again, as is known in the art, some variation in this distance can also occur.

Examples of strong constitutive promoters and root-specific promoters that are suitable for use in expressing phytase in the roots of plants include those listed in Table 2, amongst others. The promoters listed in Table 2 are provided for the purposes of exemplification only and the present invention is not to be limited by the list provided therein. Those skilled in the art will readily be in a position to provide additional promoters that are useful in performing the present invention.

Preferred tissue-specific inducible promoter sequences include the anoxia-inducible and hypoxia-inducible maize *Adh1* gene promoter (Howard *et al.*, 1987; Walker *et al.*, 1987). Such environmentally-inducible promoters are reviewed in detail by Kuhlemeier *et al.* (1987).

Preferred chemically-inducible promoters include the 3-β- indoylacrylic acid-inducible *Tip* promoter; IPTG-inducible *lac* promoter; phosphate-inducible promoter; L-arabinose-inducible *araB* promoter; heavy metal-inducible metallothionine gene promoter; dexamethasone-inducible promoter; glucocorticoid-inducible promoter; ethanol-inducible promoter (Zeneca); the N,N-diallyl-2,2-dichloroacetamide-inducible glutathione-S-transferase gene promoter (Wiegand *et al.*, 1986); or any one or more of the chemically-inducible promoters described by Gatz *et al.* (1996;1998), amongst others.

Preferred wound-inducible or pathogen-inducible promoters include the phenylalanine ammonia lyase (PAL) gene promoter (Ebel *et al.*, 1984), chalcone synthase gene promoter (Ebel *et al.*, 1984) or the potato wound-inducible promoter (Cleveland *et al.*, 1987), amongst others.

In the case of constitutive promoters or promoters that induce expression throughout the entire plant, such sequences may be modified by the addition of nucleotide sequences derived from one or more of the root-specific promoters listed in Table 2, and optionally, additional nucleotide sequences derived from one or more inducible promoters, to confer inducible tissue-specificity thereon. For example, the CaMV 35S promoter may be modified by the addition of maize *Adh1* promoter sequence, to confer

anaerobically-regulated root-specific expression thereon, as described previously (Ellis *et al.*, 1987). Such modifications can be achieved by routine experimentation by those skilled in the art.

In a particularly preferred embodiment of the present invention, the phytase is ectopically expressed under control of the CaMV 35S promoter sequence.

In each of the preceding embodiments of the present invention, the phytase protein or a homologue, analogue, or derivative thereof, in particular the *A. niger* phytase protein phyA or 9-1, is expressed under the operable control of a promoter sequence operable in the root. As will be known those skilled in the art, this is generally achieved by introducing a gene construct or vector into plant cells by transformation or transfection means. The nucleic acid molecule or a gene construct comprising same may be introduced into a cell using any known method for the transfection or transformation of said cell. Wherein a cell is transformed by the gene construct of the invention, a whole organism may be regenerated from a single transformed cell, using methods known to those skilled in the art.

TABLE 2

EXEMPLARY PROMOTERS FOR USE IN THE PERFORMANCE OF THE PRESENT INVENTION

GENE SOURCE	EXPRESSION PATTERN	REFERENCE
root-expressible genes	roots	Tingey *et al.* (1987); An *et al.* (1988);
tobacco auxin-inducible gene	root tip	Van der Zaal *et al.* (1991)
β-tubulin	root	Oppenheimer *et al.* (1988)
tobacco root-specific genes	root	Conkling *et al.* (1990)
B. napus G1-3b gene	root	United States Patent No. 5, 401, 836
SbPRP1	roots	Suzuki *et al.* (1993)
AtPRP1; AtPRP3	roots; root hairs	http://salus.medium.edu/mmg/tierney/html
RD2 gene	root cortex	http://www2.cnsu.edu/ncsu/research
TobRB7 gene	root vasculature	http://www2.cnsu.edu/ncsu/research
Actin	constitutive	
CaMV 35S	constitutive	Odell *et al* (1985)
CaMV 19S	constitutive	
Octopine synthase (OCS)	constitutive	Koncz *et al.*, (1983)
Nopaline Synthase (NOS)	constitutive	Depicker *et al.*, (1982)
gos2	constitutive	de Pater *et al.* (1992)
UBQ1	constitutive	Callis *et al.*, (1990)

INDUCIBLE PROMOTERS	INDUCTANT	REFERENCE
P5CS (delta(1)-pyrroline-5-carboxylate syntase)	salt, water	Zhang *et al* (1997)
cor15a	cold	Hajela *et al* (1990)
cor15b	cold	Wilhelm *et al* (1993)
cor15a (-305 to +78 nt)	cold, drought	Baker *et al* (1994)
rd29	salt, drought, cold	Kasuga *et al* (1999)
heat shock proteins, including artificial promoters containing the heat shock element (HSE)	heat	Barros *et al* (1992); Marrs *et al* (1993); Schoffl *et al* (1989)
smHSP (small heat shock proteins)	heat	Waters *et al.* (1989)
wcs120	cold	Oullet *et al* (1998)
ci7	cold	Kirch *et al* (1997)
Adh	cold, drought, hypoxia	Dolferus *et al*(1994)
pwsi18	water: salt and drought	Joshee *et al* (1998)
ci21A	cold	Schneider *et al* (1997)
Trg-31	drought	Chaudhury *et al* (1996)
osmotin	osmotic stress	Raghothama *et al* (1993)

By "transfect" is meant that the gene construct or vector or an active fragment thereof comprising the *PhyA* or *9-1* gene or a homologue, analogue or derivative thereof, operably under the control of the promoter sequence is introduced into said cell without integration into the cell's genome.

By "transform" is meant that the gene construct or vector or an active fragment thereof comprising the *PhyA* or *9-1* gene or a homologue, analogue or derivative thereof, operably under the control of the plant-expressible promoter sequence is stably integrated into the genome of the cell.

In an alternative embodiment, the present invention provides a method of improving the phosphorus nutrition of a plant comprising:

(i) ectopically expressing in the root of a plant an isolated nucleic acid molecule encoding a phytase polypeptide for a time and under conditions sufficient for said phytase to be secreted from the root; and

(ii) modifying the chemistry of the soil around the root or other growth medium around the root for a time and under conditions sufficient to solubilise phosphorus produced by the action of said phytase enzyme on phytate.

As used herein, the term "modifying the chemistry of the soil around the root or other growth medium around the root using an organic acid" or similar shall be taken to include any effect of an organic acid on increasing the ability of a plant to utilise phytase-labile phosphorus and/or total organic phosphorus, such as, for example, acidification, and/or a chelating effect that facilitates the solubilisation of phosphorus and/or phosphorus uptake, amongst others. The present invention is not to be limited by the mode of action of the organic acid in improving phosphorus nutrition, the only requirement being that the amount of organic acid in the vicinity of the root is increased, such as by direct application, extracellular secretion or active transport, amongst others.

The present invention particularly extends to the use of any agent known to those

skilled in the art to modify the chemistry of the soil by chelation, preferably by chelation of a metal, such as, for example a transition metal, to facilitate phosphorus uptake by a plant. In this regard, phosphorus in the soil which is released by the breakdown of phytate may form complexes with various metals in the soil, such as, for example, aluminium. Without being bound by any theory or mode of action, the presence of an organic acid in the vicinity of the root where phytase acts in accordance with the inventive method may chelate those aluminium and/or iron and/or calcium deposits in the soil that would otherwise bind phosphorus, thereby ensuring that soluble phosphorus is maintained in the soil for absorption by the plant.

According to this embodiment, the chemistry of the soil around the root may be altered in accordance with the present invention by any means known to those skilled in the art, including the application of organic acids to the soil or growth medium, or the addition of agents known to those skilled in the art that chelate metals but not phosphorus.

However, a particularly preferred means comprises expressing an organic acid biosynthetic enzyme in the roots of the plant so as to increase the intracellular level of organic acids, and the subsequent efflux of organic acids from the root. Preferably, the expression of the organic acid biosynthetic enzyme is targeted to the same cells in which the phytase gene is expressed, to optimise the local extracellular concentrations of available phosphorus in that region of the root which is involved in phosphorus uptake.

Preferably, the organic acid biosynthesis enzyme is citrate synthase. Expression of citrate synthase may be increased in the region around the root by ectopically-expressing a citrate synthase-encoding nucleic acid molecule in the root under the control of a root-specific promoter sequence or constitutive promoter sequence as described herein, and preferably, targeting the citrate synthase polypeptide product of such expression to the root surface.

As will be apparent to those skilled in the art, the ectopic expression of citrate synthase and targeting of the citrate synthase polypeptide to the root surface may be performed in a similar manner to the expression and secretion of the phytase polypeptide, in accordance with the description provided herein. Preferably, in the performance of this embodiment of the invention, the nucleic acid molecules encoding phytase and the organic acid biosynthesis enzyme are placed operably in connection with different promoter sequences, to minimise competition therebetween for nuclear transcription factors, which competition may reduce expression of one or other structural gene.

Alternatively or in addition, the chemistry of the soil or other growth medium is increased by expressing an organic acid transporter polypeptide in the roots of the plant for a time and under conditions sufficient for the rate or amount of organic acid outside the root to increase.

A further aspect of the present invention clearly provides a gene construct or vector to facilitate the ectopic expression and/or maintenance of the phytase protein-encoding sequence and promoter in a plant cell, tissue or organ.

It will be apparent from the preceding statements that the gene construct of the invention will at least comprise a phytase protein-encoding sequence, optionally further comprising nucleotide sequences encoding a secretory signal sequence operable in plant cells, and a promoter sequence operable in the root cells of a plant operably connected thereto.

The present invention clearly encompasses genetic constructs that further comprise a nucleotide sequence that encodes an organic acid biosynthesis enzyme, in particular citrate synthase, placed operably under the control of a further root-operable or constitutive promoter sequence.

Additionally, the gene construct of the present invention may further comprise one or more terminator sequences.

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3'-non-translated DNA sequences containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3'-end of a primary transcript. Terminators active in cells derived from viruses, yeasts, moulds, bacteria, insects, birds, mammals and plants are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals and/or plants.

Examples of terminators particularly suitable for use in the gene constructs of the present invention include the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene terminator, the *Agrobacterium tumefaciens* octopine synthase (OCS) gene terminator sequence, the Cauliflower mosaic virus (CaMV) 35S gene terminator sequence, the *Oryza sativa* ADP-glucose pyrophosphorylase terminator sequence (t3'Bt2), the *Zea mays zein* gene terminator sequence, the *rbcs-1A* gene terminator, and the *rbcs-3A* gene terminator sequences, amongst others.

Those skilled in the art will be aware of additional promoter sequences and terminator sequences which may be suitable for use in performing the invention. Such sequences may readily be used without any undue experimentation.

The gene constructs of the invention may further include an origin of replication sequence which is required for maintenance and/or replication in a specific cell type, for example a bacterial cell, when said gene construct is required to be maintained as an episomal genetic element (eg. plasmid or cosmid molecule) in said cell. Preferred origins of replication include, but are not limited to, the *f1*-ori and *col*E1 origins of replication.

The gene construct may further comprise a selectable marker gene or genes that are functional in a cell into which said gene construct is introduced.

As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or

selection of cells which are transfected or transformed with a gene construct of the invention or a derivative thereof.

Suitable selectable marker genes contemplated herein include the ampicillin resistance (Amp^r), tetracycline resistance gene (Tc^r), bacterial kanamycin resistance gene (Kan^r), phosphinothricin resistance gene, neomycin phosphotransferase gene (*npt*II), hygromycin resistance gene, β-glucuronidase (GUS) gene, chloramphenicol acetyltransferase (CAT) gene, green fluorescent protein (*gfp*) gene (Haseloff *et al*, 1997), and luciferase gene, amongst others.

In fact, the phytase protein-encoding sequence may also be used as a selectable marker gene as defined herein, by virtue of the improved phosphorus nutrition of plants secreting phytase from their roots in accordance with the inventive method, including their ability to grow on phytate-containing media. Accordingly, the present invention clearly encompasses the selection of transformed plants by their ability to regenerate on media having phytate as the source of phosphorus.

In a further preferred embodiment, the present invention provides a method of modifying the phosphorus nutrition of a plant comprising:

(i) introducing to a plant cell, tissue or organ a gene construct or vector comprising a nucleotide sequence that encodes phytase in a secretable form, operably in connection with a promoter sequence capable of conferring expression in the roots of a plant;

(ii) regenerating a whole plant therefrom; and

(ii) expressing said phytase in the root or one or more of said cells or tissues thereof such that it is secretable to the surface of the root.

A further aspect of the invention provides a transformed plant ectopically-expressing phytase in secretable form, preferably as an in-frame fusion with a secretable signal sequence.

Means for introducing recombinant DNA into bacterial cells, yeast cells, or plant,

insect, fungal (including mould), avian or mammalian tissue or cells include, but are not limited to, transformation using $CaCl_2$ and variations thereof, in particular the method described by Hanahan (1983), direct DNA uptake into protoplasts (Krens *et al*, 1982; Paszkowski *et al*, 1984), PEG-mediated uptake to protoplasts (Armstrong *et al*, 1990), electroporation (Fromm *et al.*, 1985), microinjection of DNA (Crossway *et al.*, 1986), microparticle bombardment of tissue explants or cells (Christou *et al*, 1988; Sanford *et al.*, 1987; Finer and McMullen, 1990; Finer *et al.*, 1992; Sanford *et al.*, 1993; Karunaratne *et al.*, 1996; and Abedinia *et al.*, 1997), vacuum-infiltration of tissue with nucleic acid, or T-DNA-mediated transfer from *Agrobacterium* to the plant tissue (An *et al.*1985; Herrera-Estrella *et al.*, 1983a; 1983b; 1985).

For example, the transformed plants can be produced by the method the *in planta* transformation method using *Agrobacterium tumefaciens* (Bechtold *et al.*,1993; Clough *et al.*,1998), wherein *A. tumefaciens* is applied to the outside of the developing flower bud and the binary vector DNA is then introduced to the developing microspore and/or macrospore and/or the developing seed, so as to produce a transformed seed. Those skilled in the art will be aware that the selection of tissue for use in such a procedure may vary, however it is preferable generally to use plant material at the zygote formation stage for *in planta* transformation procedures.

Alternatively, microparticle bombardment of cells or tissues may be used, particularly in cases where plant cells are not amenable to transformation mediated by *A. tumefaciens*. In such procedures, microparticle is propelled into a cell to produce a transformed cell. Any suitable ballistic cell transformation methodology and apparatus can be used in performing the present invention. Exemplary apparatus and procedures are disclosed by Stomp *et al.* (U.S. Patent No. 5,122,466) and Sanford and Wolf (U.S. Patent No. 4,945,050). When using ballistic transformation procedures, the genetic construct may incorporate a plasmid capable of replicating in the cell to be transformed.

Examples of microparticles suitable for use in such systems include 1 to 5 μm gold

spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

A whole plant may be regenerated from the transformed or transfected cell, in accordance with procedures well known in the art. Plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a gene construct of the present invention and a whole plant regenerated therefrom. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

The term "organogenesis", as used herein, means a process by which shoots and roots are developed sequentially from meristematic centres.

The term "embryogenesis", as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes.

The generated transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plant may be selfed to give homozygous second generation (or T2) transformant, and the T2 plants further propagated through classical breeding techniques.

The generated transformed organisms contemplated herein may take a variety of forms. For example, they may be chimeras of transformed cells and non-transformed cells; clonal transformants (e.g., all cells transformed to contain the expression cassette); grafts of transformed and untransformed tissues (e.g., in plants, a transformed root stock grafted to an untransformed scion).

The present invention is applicable to any plant, in particular a monocotyledonous plants and dicotyledonous plants including a fodder or forage legume, companion plant, food crop, tree, shrub, or ornamental selected from the list comprising *Acacia spp., Acer spp., Actinidia spp.,Aesculus spp., Agathis australis, Albizia amara, Alsophila tricolor, Andropogon spp., Arabidopsis thaliana, Arachis spp, Areca catechu, Astelia fragrans, Astragalus cicer, Baikiaea plurijuga, Betula spp., Brassica spp., Bruguiera gymnorrhiza, Burkea africana, Butea frondosa, Cadaba farinosa, Calliandra spp, Camellia sinensis, Canna indica, Capsicum spp., Cassia spp., Centroema pubescens, Chaenomeles spp.,Cinnamomum cassia, Coffea arabica, Colophospermum mopane, Coronillia varia, Cotoneaster serotina, Crataegus spp., Cucumis spp., Cupressus spp., Cyathea dealbata, Cydonia oblonga, Cryptomeria japonica, Cymbopogon spp., Cynthea dealbata, Cydonia oblonga, Dalbergia monetaria, Davallia divaricata, Desmodium spp., Dicksonia squarosa, Diheteropogon amplectens, Dioclea spp, Dolichos spp., Dorycnium rectum, Echinochloa pyramidalis, Ehrartia spp., Eleusine coracana, Eragrestis spp., Erythrina spp., Eucalyptus spp., Euclea schimperi, Eulalia villosa, Fagopyrum spp., Feijoa sellowiana, Fragaria spp., Flemingia spp, Freycinetia banksii, Geranium thunbergii, Ginkgo biloba, Glycine javanica, Gliricidia spp, Gossypium hirsutum, Grevillea spp., Guibourtia coleosperma, Hedysarum spp., Hemarthia altissima, Heteropogon contortus, Hordeum vulgare, Hyparrhenia rufa, Hypericum erectum, Hyperthelia dissoluta, Indigo incarnata, Iris spp., Leptarrhena pyrolifolia, Lespediza spp., Lettuca spp., Leucaena leucocephala, Loudetia simplex, Lotonus bainesii, Lotus spp., Macrotyloma axillare, Malus spp., Manihot esculenta, Medicago sativa, Metasequoia glyptostroboides, Musa sapientum, Nicotianum spp., Onobrychis spp., Ornithopus spp., Oryza spp., Peltophorum africanum, Pennisetum spp., Persea gratissima, Petunia spp., Phaseolus spp., Phoenix canariensis, Phormium cookianum, Photinia spp., Picea glauca, Pinus spp., Pisum sativum, Podocarpus totara, Pogonarthria fleckii, Pogonarthria squarrosa, Populus spp., Prosopis cineraria, Pseudotsuga menziesii, Pterolobium stellatum, Pyrus communis, Quercus spp., Rhaphiolepsis umbellata, Rhopalostylis sapida, Rhus natalensis, Ribes grossularia, Ribes spp., Robinia pseudoacacia, Rosa spp., Rubus*

spp., Salix spp., Schyzachyrium sanguineum, Sciadopitys verticillata, Sequoia sempervirens, Sequoiadendron giganteum, Sorghum bicolor, Spinacia spp., Sporobolus fimbriatus, Stiburus alopecuroides, Stylosanthos humilis, Tadehagi spp, Taxodium distichum, Themeda triandra, Trifolium spp., Triticum spp., Tsuga heterophylla, Vaccinium spp., Vicia spp.Vitis vinifera, Watsonia pyramidata, Zantedeschia aethiopica, Zea mays, rice, straw, amaranth, onion, asparagus, sugar cane, soybean, sugarbeet, sunflower, carrot, celery, cabbage, canola, tomato, potato, lentil, flax, broccoli, oilseed rape, cauliflower, brussel sprout, artichoke, okra, squash, kale, collard greens, and tea, amongst others, or the seeds of any plant specifically named above or a tissue, cell or organ culture of any of the above species.

Preferably, the plant is a plant that is capable of being transfected or transformed with a genetic sequence, or which is amenable to the introduction of a protein by any art-recognised means, such as microprojectile bombardment, microinjection, *Agrobacterium-mediated* transformation, protoplast fusion, protoplast transformation, *in planta* transformation, or electroporation, amongst others.

This aspect of the invention further extends to plant cells, tissues, organs and plants parts, propagules and progeny plants of the primary transformed or transfected cells, tissues, organs or whole plants that also comprise the introduced isolated nucleic acid molecule or gene construct comprising same, and, as a consequence, exhibit similar phenotypes to the primary transformants/transfectants or at least are useful for the purpose of replicating or reproducing said primary transformants/transfectants.

A further aspect of the invention provides an isolated nucleic acid molecule comprising a nucleotide sequence which encodes or is complementary to a nucleotide sequence which encodes a phytase peptide, oligopeptide, polypeptide, protein or enzyme having at least about 93% nucleotide sequence identity to the *Aspergillus niger 9-1* gene sequence set forth in SEQ ID NO: <400> 1 or SEQ ID NO: <400> 9 or a complementary nucleotide sequence thereto.

Preferably, the percentage identity to SEQ ID NO: <400> 1 or SEQ ID NO: <400> 9

is at least about 95%, more preferably at least about 97%, and still more preferably at least about 99%. In a particularly preferred embodiment, the isolated nucleic acid molecule of the invention comprises or contains the nucleotide sequence set forth in SEQ ID NO:<400> 1 or SEQ ID NO: <400> 9 or a fragment thereof that encodes an enzymically-functional phytase peptide, oligopeptide or polypeptide.

In determining whether or not two nucleotide or amino acid sequences fall within defined percentage identity or similarity limits referred to herein, those skilled in the art will be aware that it is necessary to conduct a side-by-side comparison. In such comparisons or alignments, differences will arise in the positioning of non-identical residues depending upon the algorithm used to perform the alignment. In the present context, references to percentage identities between two or more sequences shall be taken to refer to the number of identical and similar residues respectively, between said sequences as determined using any standard algorithm known to those skilled in the art. In particular, nucleotide and/or amino acid identities can be calculated using the GAP programme of the Computer Genetics Group, Inc., University Research Park, Madison, Wisconsin, United States of America (Devereaux *et al*, 1984), which utilizes the algorithm of Needleman and Wunsch (1970) or alternatively, the CLUSTAL W algorithm of Thompson *et al* (1994) for multiple alignments, to maximise the number of identical/similar residues and to minimise the number and/or length of sequence gaps in the alignment.

Alternatively or in addition, the present invention encompasses those phytase-encoding nucleotide sequences that are capable of hybridising under high stringency hybridisation conditions to SEQ ID NO: <400> 1 or SEQ ID NO: <400> 9 or a complementary nucleotide sequence thereto, but not including the *phyA* gene sequence.

For the purposes of defining the level of stringency, those skilled in the art will be aware that several different hybridisation conditions may be employed. As used herein, a high stringency may comprise a standard reaction buffer used in a polymerase chain reaction (PCR) to anneal an oligonucleotide primer to template DNA

at temperatures higher than 42°C, or alternatively, a standard DNA/DNA hybridisation and/or wash carried out in 0.1xSSC buffer, 0.1% (w/v) SDS at a temperature of at least 65°C, or equivalent annealing/hybridisation conditions.

As will be known to those skilled in the art, the stringency is increased by reducing the concentration of SSC buffer, and/or increasing the concentration of SDS in a standard hybridisation, and/or increasing the temperature of the annealing/hybridisation of PCR or a standard hybridisation, and/or increasing the temperature of the wash in a standard hybridisation. Conditions for hybridisations and washes are well understood by one normally skilled in the art. For the purposes of clarification (to parameters affecting hybridisation between nucleic acid molecules), reference is found in pages 2.10.8 to 2.10.16. of Ausubel *et al.* (1987), which is herein incorporated by reference.

As will be known to those skilled in the art, the specificity of PCR may also be increased by reducing the number of cycles, or the time per cycle, or by the use of specific PCR formats, such as, for example, a nested PCR, a format that is well-known to those skilled in the art. For the purposes of clarification of the parameters affecting the specificity of PCR, reference is made herein to McPherson *et al.* (1991) which is incorporated by way of reference.

Particularly preferred variants of the *A. niger 9-1* gene exemplified herein comprise degenerate nucleotide sequences (i.e. homologues) that encode the amino acid sequence set forth in SEQ ID NO: <400> 2 or SEQ ID NO: <400> 10.

Preferably, the isolated nucleic acid molecule encodes a phytase polypeptide, protein or enzyme as an in-frame fusion with a secretory signal peptide, in particular the carrot extensin signal peptide.

Homologues, analogues and derivatives of the phytase-encoding nucleotide sequence of the present invention may be obtained by any standard procedure known to those skilled in the art, such as by nucleic acid hybridization (Ausubel *et al*, 1987),

polymerase chain reaction (McPherson *et al*, 1991) screening of expression libraries using antibody probes (Huynh *et al*, 1985), and the invention encompasses all such homologues, analogues and derivatives falling within the above-mentioned sequence identity and/or hybridisation limitations.

In nucleic acid hybridizations, genomic DNA, mRNA or cDNA or a part of fragment thereof, in isolated form or contained within a suitable cloning vector such as a plasmid or bacteriophage or cosmid molecule, is contacted with a hybridization-effective amount of a nucleic acid probe derived from SEQ ID NO: <400> 1 or SEQ ID NO: <400> 9 for a time and under conditions sufficient for hybridization to occur and the hybridized nucleic acid is then detected using a detecting means.

Detection is performed preferably by labelling the probe with a reporter molecule capable of producing an identifiable signal, prior to hybridization. Preferred reporter molecules include radioactively-labelled nucleotide triphosphates and biotinylated molecules.

Preferably, variants of the *A. niger 9-1* gene exemplified herein, including genomic equivalents, are isolated by hybridisation under high stringency conditions, to the probe.

In the polymerase chain reaction (PCR), a nucleic acid primer molecule comprising at least about 14 nucleotides in length derived from the *A. niger 9-1* gene is hybridized to a nucleic acid template molecule and specific nucleic acid molecule copies of the template are amplified enzymatically as described in McPherson *et al*, (1991), which is incorporated herein by reference.

In expression screening of cDNA libraries or genomic libraries, protein- or peptide-encoding regions are placed operably under the control of a suitable promoter sequence in the sense orientation, expressed in a prokaryotic cell or eukaryotic cell in which said promoter is operable to produce a peptide or polypeptide, screened with a monoclonal or polyclonal antibody molecule or a derivative thereof against one or more

epitopes of a phytase polypeptide and the bound antibody is then detected using a detecting means, essentially as described by Huynh *et al* (1985) which is incorporated herein by reference. Suitable detecting means according to this embodiment include ^{125}I-labelled antibodies or enzyme-labelled antibodies capable of binding to the first-mentioned antibody, amongst others.

A still further aspect of the present invention provides an isolated or recombinant phytase polypeptide selected from the group consisting of:

(i) a phytase polypeptide having at least 95% amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO: <400> 2 or SEQ ID NO: <400> 10;

(ii) a phytase polypeptide encoded by a nucleotide sequence having at least 93% identity to SEQ ID NO: <400> 1 or SEQ ID NO: <400> 9;

(iii) fragments of (i) or (ii) that possess phytase enzyme activity;

(iv) in-frame fusion polypeptides comprising (i) and/or (ii) and/or (iii) linked to a secretory signal peptide, in particular the carrot extensin secretory signal peptide.

It will be apparent from the preceding description that a recombinant phytase polypeptide or an in-frame fusion polypeptide comprising same may be produced by standard means by expressing a phytase-encoding nucleotide sequence operably under the control of a suitable promoter sequence in a host cell for a time and under conditions sufficient for translation to occur. Such expression may be carried out in a prokaryotic cell, such as, for example, a bacterial cell. Alternatively, such expression may be performed in a eukaryotic cell such as an insect cell, mammalian cell, plant cell, fungal cell, or yeast cell, amongst others. In any case, unless the sense molecule is expressed under the control of a strong universal promoter, it is important to select a promoter sequence which is capable of regulating expression in the cell comprising the said nucleic acid molecule in an expressible format. Persons skilled in the art will be in a position to select appropriate promoter sequences for expression of the sense molecule without undue experimentation.

Examples of promoters useful in performing this embodiment include the CaMV 35S promoter, NOS promoter, octopine synthase (OCS) promoter, *Arabidopsis thaliana SSU*gene promoter, napin seed-specific promoter, P_{32} promoter, BK5-T *imm* promoter, *lac* promoter, *tac* promoter, phage lambda λ_L or λ_R promoters, CMV promoter (U.S. Patent No. 5,168,062), T7 promoter, lacUV5 promoter, SV40 early promoter (U.S. Patent No. 5,118,627), SV40 late promoter (U.S. Patent No. 5,118,627), adenovirus promoter, baculovirus P10 or polyhedrin promoter (U.S. Patent Nos. 5,243,041, 5,242,687, 5,266,317, 4,745,051 and 5,169,784), and the like. In addition to the specific promoters identified herein, cellular promoters for so-called housekeeping genes are useful.

In a preferred embodiment, the recombinant phytase polypeptide is provided in a sequencably-pure format or a pure format substantially free of conspecific proteins.

By "sequencably pure" is meant that the subject polypeptide or a homologue, analogue, derivative or epitope thereof is purified sufficiently to facilitate amino acid sequence determination.

Preferably, said polypeptide or a homologue, analogue, derivative or epitope is at least about 20% pure, more preferably at least about 40% pure, even more preferably at least about 60% pure and even more preferably at least about 80% pure or 95% pure on a weight basis.

For the purposes of describing the present invention in more detail, a plasmid comprising *a. niger 9-1* gene as an in-frame fusion with the carrot extensin secretion signal-encoding nucleotide sequence, and operably in connection with the CaMV 35S promoter, was deposited on 23 September, 1999, with the Australian Government Analytical Laboratories (AGAL) at 1, Suakin Street Pymble, New South Wales 2073, Australia, under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, and accorded AGAL Accession No. NM99/06795. Accordingly, the presently-described invention clearly extends to the use of the deposited plasmid and/or the phytase-encoding

portion thereof or variants thereof, with or without the secretory signal-encoding portion of said plasmid, in accordance with the scope of each and every embodiment described herein.

The present invention is further described with reference to the following non-limiting Examples and to the drawings.

EXAMPLE 1

Ectopic expression of phytase under control of the CaMV 35S promoter

I. The *PhyA* gene

The *PhyA* gene was originally obtained from Dr Mullaney (United States Department of Agriculture) as a 7.016 kb plasmid (pMD4.21), containing a 2.7 kb *Sph*I clone of the *Aspergillus ficuum* strain NRRL3135 (now termed *A. niger*) *phyA* gene (termed CDS), cloned into the plasmid vector pBR322.

The 2.7 kb insert contained a genomic clone of the *phyA* gene with 5' and 3' flanking sequences, including an *Aspergillus* 5' leader sequence and an intron (102 bp) upstream of the coding region for the mature protein (Genbank Accession No. M94550; Van Hartingsveldt *et al.*,1993).

Modifications were made to the *PhyA* gene, to delete the leader sequence and the intron, using PCR. These modifications introduced a "new" ATG translation start in the open reading frame, immediately prior to, and in frame with, the nucleotide sequence for the mature peptide. The stop codon (CTA[TAG in reverse strand]) in the reverse primer is identical to the published gene.

The sequence of the PCR primers (which also contained cloning sites for *Eco*RI and *Cla*I, respectively; underlined lower case) to obtain a modified version of the gene that is functional in plants were as follows:

Forward: (PHYF2): cgcgaattcATG CTGGCAGTCCCCGCCTCG

Reverse: (PHYR3): ggcatcgatCTAAGCAAAACACTCCGC

II. The 9-1 (phytase) gene

The PCR primers PHYF2 and PHYR3 were used to amplify a derivative of the *phyA* gene from genomic DNA isolated from *Aspergillus niger*, strain ATCC9029. This gene has been designated " 9-1". The 9-1 gene has approximately 92% DNA sequence identity to the *phyA* gene. Additionally, there is about 95% amino acid sequence identity between PhyA and 9-1 polypeptides (Figures 1 and 2).

III. The Carrot extensin secretory signal sequence.

Nucleotide sequences encoding the secretory signal sequence of the carrot extensin gene was amplified using PCR, from plasmid pSEGON, obtained from D. Llewellyn, Commonwealth Scientific and Industrial Research Organisation, Canberra, Australia, which plasmid contains nucleotide sequences encoding the extensin signal peptide upstream of a glucose oxidase-encoding gene.

The PCR primers used to amplify the extensin secretory signal sequence were as follows, wherein cloning sites in the primers are underlined and in lower case:

Forward: gcgtctagagaattcATGGGAAGAATTGCTAG; and

Reverse: cgcggatccgcggccgcAGCTGTGGTTTCGGAAGC.

The amplified product was first subcloned as a *Xba*I/*Bam*HI fragment into plasmid pBSIIKS (Stratagene, Palo Alto, California, USA). The amplified fragment was 99 bp in length and codes for 33 amino acids. The sequence of the leader is set forth in SEQ ID NO: <400> 5.

IV. Extensin/Phytase Gene Fusion

"In-frame" fusions between the extensin secretory signal-encoding sequence and both the *phyA* and *9-1* genes were generated by ligation between the *Not*I site located at the 3' end of the extension secretory signal-encoding sequence and the *Eco*RI site at eh 5' end of the *phA* and 9-1 genes. This was achieved by generating blunt (ie.,

flushed) ends of the restriction enzymes sites prior to ligation.

The junction sequence generated from the extensin/phytase fusion comprised the nucleotide sequence 5′-ACGCTGCCATGCTGGCA-3′, encoding the junction amino acid sequence Thr-Ala-Ala-Met-Leu-Ala, which amino acid sequence comprises two codons derived from extensin, an inserted alanine (encoded by the *Not*I-*Eco*RI fusion), and three codons derived from the phytase genes (*phyA* or 9-1 as appropriate).

Without being bound by any theory or mode of action, the Alanine residue at the extensin::phytase junction provides a suitable site for protease cleavage of the precursor polypeptide to remove the extensin secretory signal sequence.

The extensin/phytase in-frame fusions were generated and verified in plasmid pBSIIKS.

V. Construction of vectors for plant transformation

Four gene expression cassettes were produced, comprising the *phyA* gene, chimeric *extensin::phyA* gene, *9-1* gene, and chimeric *extensin::9-1* gene, by subcloning these genes, as *Eco*RI-*Kpn*I fragments, from their plasmid pBSIIKS vector (see above) into plasmid pART7 (Figure 4), which contains a CaMV 35S promoter and *ocs* terminator.

Accordingly, in the resultant plasmid constructs, the phytase genes were placed in operable connection with the CaMV 35S promoter sequence, to produce the plasmids:

1. *35S+phyA+ocs;*
2. *35S+extensin::phyA+ocs;*
3. *35S+9-1+ocs;* and
4. *35S+extensin::9-1+ocs.*

The four phytase transgenes were then subcloned as *Not*I fragments into the binary vector pBS389 (Figure 3). This vector is suitable for plant transformation and contains the selectable marker *npt*II under control of the SCSV Sc1 promoter and Sc3

terminator sequences. The phytase transgenes were cloned such that their orientation (determining the direction of transcription) was the same as the orientation as the selectable marker.

VI. Plant transformation

The pBS389 vectors containing the various phytase gene constructs were transferred to *Agrobacterium tumefaciens strain* AGL1 using standard, tri-parental mating techniques. These strains were used to transform tobacco, *Arabidopsis* and subterranean clover using published protocols. Transformed plants of all three species were generated and have been verified to contain the various phytase transgenes by selection on Kanamycin, PCR an/or northern blot analysis using the *phyA* gene as a hybridisation probe.

VII. Analysis of transformed plants

A) Production of phytase-encoding mRNA

Northern blot hybridisations have been conducted to confirm ectopic expression of the introduced phytase genes transgenic tobacco, *Arabidopsis* and subterranean clover plants.

Data obtained indicated that, in most cases, highest levels of phytase-encoding mRNA were present in plants that contain the phytase genes (*PhyA* or *9-1*) fused to the extensin secretory signal-encoding nucleotide sequence, suggesting that these sequences may also increase mRNA stability.

b) Phytase enzyme activity

Phytase enzyme assays were performed using leaf material derived from transformed tobacco plants.

The phytase activity in leaf extracts of control plants was 0.24 nkat phytase g^{-1} fresh wt (0,04 nkat phytase mg^{-1} protein), compared to 30.4 nkat g^{-1} fresh wt (6.2 nkat phytase mg^{-1} protein) for transformed plants containing the *extensin::phyA* gene

construct, representing a 130-fold increase in enzyme activity. Similar results are obtained for phytase assays of roots.

Similarly, phytase enzyme activity increases in the leaves and roots of transgenic *A. thaliana* and clover that contain the introduced phytase gene constructs.

c) *Plant Growth on phytate-containing media*

The growth responses of transgenic plants in media supplemented with phytate were determined for transformed *A. thaliana* plants. Data shown in Figure 7 show that plants ectopically-expressing the extensin-phytase fusion polypeptide grow more rapidly on media containing phytate as a sole source of phosphorus. Significantly more pant growth was also obtained for plants expressing the extensin-phytase fusion polypeptide compared to plants expressing phytase but unable to target the phytase to the extracellular space (Figure 8).

EXAMPLE 2

Phytase activities of plant roots and localisation of activity

The phytase activities in the roots of a wide range of agriculturally important legume and grass species, including subterranean clover, burr medic, white clover, lucerne, tobacco, *Arabidopsis,* wheat, phalaris, ryegrass and danthonia, have been determined.

In summary, phytase activities in extracts prepared from roots of these species ranged between 0.1 and 1.7 nkat g^{-1} root fresh wt (0.2 to 1.5 nkat mg^{-1} total protein), with levels of activity increased by up to 3.3-fold (9.8-fold on a total root protein basis) when seedlings were grown in conditions of phosphorus deficiency.

In contrast, acid phosphatase activity measured in the same extracts ranged between 20 and 60 nkat g^{-1} root fresh wt. Phytase activity was a small component only (less than 5% for the range of species investigated) of the total acid phosphatase activity of plant roots, irrespective of the level of phosphorus nutrition.

The extracellular component of root phytase activity is a minor proportion of the total phytase activity measurable in the roots of naturally-occurring plants. For example, less than 0.042 nkat phytase activity g^{-1} root fresh wt (ie., less than 4.7% of the activity measured in root extracts) could be eluted from roots of subterranean clover. The estimated extracellular root phytase activity of intact roots of subterranean clover seedlings is as low as 0.03 nkat g^{-1} root fresh wt (ie., 3% or less of the activity measured in soluble root extracts).

However, supplementation of subterranean clover roots with phytase, at a rate that is equivalent to -0.13 nkat g^{-1} root fresh wt, resulted in a significant enhancement of the ability of the plants to acquire phosphorus from phytate.

EXAMPLE 3

Effect of organic acids on the phytase-labile component of soil phosphorus

We have also demonstrated that the efficacy of organic acids in improving the extractability of phosphorus from the soil, and to show that a significant component of the organic P in soil actually extracted by citrate is amenable to dephosphorylation by phytase (Figure 9).

Briefly, extracts were prepared from two soils using water, or up to 50 mM citric acid as the liquid phase. Soil extracts were then incubated with *A. niger* phytase preparations that were either (i) highly specific against phytate or (ii) a commercial phytase that was active against a broad range of substrates. The phytases were added at 2.28 and 0.50 nkat g^{-1} soil, respectively.

The citric acid extracts of soil contained up to 25.3 μg organic phosphorus g^{-1} soil. A large proportion (up to 79%) of this organic phosphorus was hydrolysed by the commercial phytase, while up to 40% was hydrolysed using purified phytase. By comparison, only small quantities of the organic phosphorus in water extracts were enzyme-labile (less than 17 and 8%, respectively).

While extractable organic phosphorus was increased both with increasing

concentrations of citric acid and increasing pH of the citrate preparations (pH 2.3 to pH 6.0), phytase-labile phosphorus increased only with citric acid concentration (Figure 9). Phytase-labile phosphorus could not be extracted using HCl as an extractant, suggesting that the observed responses are associated with chelation rather than acidification.

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AND
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Met Gln Cys Gln Ser Glu Gln Glu Pro Leu Val Arg Val Leu Val Asn
            340                 345                 350

gat cgt gtt gtt ccg ctg cat ggc tgt ccg gtt gat gct ttg gga aga   1104
Asp Arg Val Val Pro Leu His Gly Cys Pro Val Asp Ala Leu Gly Arg
        355                 360                 365

tgt acg cgg gat agc ttc gtg aag ggg ttg agc ttt gcc aga tct ggg   1152
Cys Thr Arg Asp Ser Phe Val Lys Gly Leu Ser Phe Ala Arg Ser Gly
    370                 375                 380

ggt gat tgg gcg gag tgt ttt gct tag                              1179
Gly Asp Trp Ala Glu Cys Phe Ala
385                 390


<210> 2
<211> 392
<212> PRT
<213> Aspergillus niger

<400> 2
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  1               5                  10                  15
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20 25 30

Tyr Ala Pro Phe Phe Ser Leu Ala Asn Lys Ser Ala Ile Ser Pro Asp
35 40 45

Val Pro Ala Gly Cys His Val Thr Phe Ala Gln Val Leu Ser Arg His
50 55 60

Gly Ala Arg Tyr Pro Thr Asp Ser Lys Gly Lys Lys Tyr Ser Ala Leu
65 70 75 80

Ile Glu Glu Ile Gln Gln Asn Ala Thr Thr Phe Glu Gly Lys Tyr Ala
85 90 95

Phe Leu Lys Thr Tyr Asn Tyr Ser Leu Gly Ala Asp Asp Leu Thr Pro
100 105 110

Phe Gly Glu Gln Glu Leu Val Asn Ser Gly Val Lys Phe Tyr Gln Arg
115 120 125

Tyr Glu Ser Leu Thr Arg Asn Ile Val Pro Phe Ile Arg Ser Ser Gly
130 135 140

Ser Asn Arg Val Ile Ala Ser Gly Asn Lys Phe Ile Glu Gly Phe Gln
145 150 155 160

Ser Thr Lys Leu Lys Asp Pro Arg Ala Gln Pro Gly Gln Ser Ser Ala
165 170 175

Lys Ile Asp Val Val Ile Ser Glu Ala Ser Thr Ser Asn Asn Thr Thr
180 185 190

Thr Ile Ser Thr Ser Thr Val Asp Thr Lys Leu Ser Pro Phe Cys Asp
195 200 205

Leu Phe Thr His Glu Glu Trp Ile Asn Tyr Asp Tyr Leu Gln Ser Pro
210 215 220

Asn Lys Tyr Tyr Gly His Gly Ala Gly Asn Pro Leu Gly Pro Thr Gln
225 230 235 240

Gly Val Gly Tyr Ala Asn Glu Leu Ile Ala Arg Leu Thr His Ser Pro
245 250 255

Val His Asp Asp Thr Ser Ser Asn His Thr Leu Asp Ser Asn Pro Ala
260 265 270

Thr Phe Pro Leu Asn Ser Thr Leu Tyr Ala Asp Phe Ser His Asp Asn
275 280 285

Gly Ile Ile Ser Ile Leu Phe Ala Leu Gly Leu Tyr Asn Gly Thr Lys
290 295 300

Pro Leu Ser Ser Thr Thr Ala Glu Asn Ile Thr Gln Thr Asp Gly Phe
305 310 315 320

Ser Ser Ala Trp Thr Val Pro Phe Ala Ser Arg Met Tyr Val Glu Met
325 330 335

Met Gln Cys Gln Ser Glu Gln Glu Pro Leu Val Arg Val Leu Val Asn
340 345 350

Asp Arg Val Val Pro Leu His Gly Cys Pro Val Asp Ala Leu Gly Arg
355 360 365

Cys Thr Arg Asp Ser Phe Val Lys Gly Leu Ser Phe Ala Arg Ser Gly

```
    370                 375                 380

Gly Asp Trp Ala Glu Cys Phe Ala
385                 390
```

<210> 3
<211> 1350
<212> DNA
<213> Aspergillus niger

<220>
<221> CDS
<222> (1)..(1347)

<400> 3

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Met Leu Ala Val Pro Ala Ser Arg Asn Gln Ser Ser Cys Asp Thr Val
  1               5                  10                  15

gat cag ggg tat caa tgc ttc tcc gag act tcg cat ctt tgg ggt caa   96
Asp Gln Gly Tyr Gln Cys Phe Ser Glu Thr Ser His Leu Trp Gly Gln
             20                  25                  30

tac gca ccg ttc ttc tct ctg gca aac gaa tcg gtc atc tcc cct gag   144
Tyr Ala Pro Phe Phe Ser Leu Ala Asn Glu Ser Val Ile Ser Pro Glu
         35                  40                  45

gtg ccc gcc gga tgc aga gtc act ttc gct cag gtc ctc tcc cgt cat   192
Val Pro Ala Gly Cys Arg Val Thr Phe Ala Gln Val Leu Ser Arg His
     50                  55                  60

gga gcg cgg tat ccg acc gac tcc aag ggc aag aaa tac tcc gct ctc   240
Gly Ala Arg Tyr Pro Thr Asp Ser Lys Gly Lys Lys Tyr Ser Ala Leu
 65                  70                  75                  80

att gag gag atc cag cag aac gcg acc acc ttt gac gga aaa tat gcc   288
Ile Glu Glu Ile Gln Gln Asn Ala Thr Thr Phe Asp Gly Lys Tyr Ala
                 85                  90                  95

ttc ctg aag aca tac aac tac agc ttg ggt gca gat gac ctg act ccc   336
Phe Leu Lys Thr Tyr Asn Tyr Ser Leu Gly Ala Asp Asp Leu Thr Pro
            100                 105                 110

ttc gga gaa cag gag cta gtc aac tcc ggc atc aag ttc tac cag cgg   384
Phe Gly Glu Gln Glu Leu Val Asn Ser Gly Ile Lys Phe Tyr Gln Arg
        115                 120                 125

tac gaa tcg ctc aca agg aac atc gtt cca ttc atc cga tcc tct ggc   432
Tyr Glu Ser Leu Thr Arg Asn Ile Val Pro Phe Ile Arg Ser Ser Gly
    130                 135                 140

tcc agc cgc gtg atc gcc tcc ggc aag aaa ttc atc gag ggc ttc cag   480
Ser Ser Arg Val Ile Ala Ser Gly Lys Lys Phe Ile Glu Gly Phe Gln
145                 150                 155                 160

agc acc aag ctg aag gat cct cgt gcc cag ccc ggc caa tcg tcg ccc   528
Ser Thr Lys Leu Lys Asp Pro Arg Ala Gln Pro Gly Gln Ser Ser Pro
                165                 170                 175

aag atc gac gtg gtc att tcc gag gcc agc tca tcc aac aac act ctc   576
Lys Ile Asp Val Val Ile Ser Glu Ala Ser Ser Ser Asn Asn Thr Leu
            180                 185                 190

gac cca ggc acc tgc act gtc ttc gaa gac agc gaa ttg gcc gat acc   624
Asp Pro Gly Thr Cys Thr Val Phe Glu Asp Ser Glu Leu Ala Asp Thr
        195                 200                 205
```

```
gtc gaa gcc aat ttc acc gcc acg ttc gtc ccc tcc att cgt caa cgt   672
Val Glu Ala Asn Phe Thr Ala Thr Phe Val Pro Ser Ile Arg Gln Arg
    210                 215                 220

ctg gag aac gac ctg tcc ggt gtg act ctc aca gac aca gaa gtg acc   720
Leu Glu Asn Asp Leu Ser Gly Val Thr Leu Thr Asp Thr Glu Val Thr
225                 230                 235                 240

tac ctc atg gac atg tgc tcc ttc gac acc atc tcc acc agc acc gtc   768
Tyr Leu Met Asp Met Cys Ser Phe Asp Thr Ile Ser Thr Ser Thr Val
                245                 250                 255

gac acc aag ctg tcc ccc ttc tgt gac ctg ttc acc cat gac gaa tgg   816
Asp Thr Lys Leu Ser Pro Phe Cys Asp Leu Phe Thr His Asp Glu Trp
            260                 265                 270

atc aac tac gac tac ctc cag tcc ttg aaa aag tat tac ggc cat ggt   864
Ile Asn Tyr Asp Tyr Leu Gln Ser Leu Lys Lys Tyr Tyr Gly His Gly
        275                 280                 285

gca ggt aac ccg ctc ggc ccg acc cag ggc gtc ggc tac gct aac gag   912
Ala Gly Asn Pro Leu Gly Pro Thr Gln Gly Val Gly Tyr Ala Asn Glu
    290                 295                 300

ctc atc gcc cgt ctg acc cac tcg cct gtc cac gat gac acc agt tcc   960
Leu Ile Ala Arg Leu Thr His Ser Pro Val His Asp Asp Thr Ser Ser
305                 310                 315                 320

aac cac act ttg gac tcg agc ccg gct acc ttt ccg ctc aac tct act  1008
Asn His Thr Leu Asp Ser Ser Pro Ala Thr Phe Pro Leu Asn Ser Thr
                325                 330                 335

ctc tac gcg gac ttt tcg cat gac aac ggc atc atc tcc att ctc ttt  1056
Leu Tyr Ala Asp Phe Ser His Asp Asn Gly Ile Ile Ser Ile Leu Phe
            340                 345                 350

gct tta ggt ctg tac aac ggc act aag ccg cta tct acc acg acc gtg  1104
Ala Leu Gly Leu Tyr Asn Gly Thr Lys Pro Leu Ser Thr Thr Thr Val
        355                 360                 365

gag aat atc acc cag aca gat gga ttc tcg tct gct tgg acg gtt ccg  1152
Glu Asn Ile Thr Gln Thr Asp Gly Phe Ser Ser Ala Trp Thr Val Pro
    370                 375                 380

ttt gct tcg cgt ttg tac gtc gag atg atg cag tgt cag gcg gag cag  1200
Phe Ala Ser Arg Leu Tyr Val Glu Met Met Gln Cys Gln Ala Glu Gln
385                 390                 395                 400

gag ccg ctg gtc cgt gtc ttg gtt aat gat cgc gtt gtc ccg ctg cat  1248
Glu Pro Leu Val Arg Val Leu Val Asn Asp Arg Val Val Pro Leu His
                405                 410                 415

ggg tgt ccg gtt gat gct ttg ggg aga tgt acc cgg gat agc ttt gtg  1296
Gly Cys Pro Val Asp Ala Leu Gly Arg Cys Thr Arg Asp Ser Phe Val
            420                 425                 430

agg ggg ttg agc ttt gct aga tct ggg ggt gat tgg gcg gag tgt ttt  1344
Arg Gly Leu Ser Phe Ala Arg Ser Gly Gly Asp Trp Ala Glu Cys Phe
        435                 440                 445

gct tag                                                         1350
Ala
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<210> 4
<211> 449
<212> PRT
<213> Aspergillus niger

<400> 4

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Met Leu Ala Val Pro Ala Ser Arg Asn Gln Ser Ser Cys Asp Thr Val
 1               5                  10                  15

Asp Gln Gly Tyr Gln Cys Phe Ser Glu Thr Ser His Leu Trp Gly Gln
            20                  25                  30

Tyr Ala Pro Phe Phe Ser Leu Ala Asn Glu Ser Val Ile Ser Pro Glu
        35                  40                  45

Val Pro Ala Gly Cys Arg Val Thr Phe Ala Gln Val Leu Ser Arg His
    50                  55                  60

Gly Ala Arg Tyr Pro Thr Asp Ser Lys Gly Lys Lys Tyr Ser Ala Leu
65                  70                  75                  80

Ile Glu Glu Ile Gln Gln Asn Ala Thr Thr Phe Asp Gly Lys Tyr Ala
                85                  90                  95

Phe Leu Lys Thr Tyr Asn Tyr Ser Leu Gly Ala Asp Asp Leu Thr Pro
            100                 105                 110

Phe Gly Glu Gln Glu Leu Val Asn Ser Gly Ile Lys Phe Tyr Gln Arg
        115                 120                 125

Tyr Glu Ser Leu Thr Arg Asn Ile Val Pro Phe Ile Arg Ser Ser Gly
    130                 135                 140

Ser Ser Arg Val Ile Ala Ser Gly Lys Lys Phe Ile Glu Gly Phe Gln
145                 150                 155                 160

Ser Thr Lys Leu Lys Asp Pro Arg Ala Gln Pro Gly Gln Ser Ser Pro
                165                 170                 175

Lys Ile Asp Val Val Ile Ser Glu Ala Ser Ser Ser Asn Asn Thr Leu
            180                 185                 190

Asp Pro Gly Thr Cys Thr Val Phe Glu Asp Ser Glu Leu Ala Asp Thr
        195                 200                 205

Val Glu Ala Asn Phe Thr Ala Thr Phe Val Pro Ser Ile Arg Gln Arg
    210                 215                 220

Leu Glu Asn Asp Leu Ser Gly Val Thr Leu Thr Asp Thr Glu Val Thr
225                 230                 235                 240

Tyr Leu Met Asp Met Cys Ser Phe Asp Thr Ile Ser Thr Ser Thr Val
                245                 250                 255

Asp Thr Lys Leu Ser Pro Phe Cys Asp Leu Phe Thr His Asp Glu Trp
            260                 265                 270

Ile Asn Tyr Asp Tyr Leu Gln Ser Leu Lys Lys Tyr Tyr Gly His Gly
        275                 280                 285

Ala Gly Asn Pro Leu Gly Pro Thr Gln Gly Val Gly Tyr Ala Asn Glu
    290                 295                 300

Leu Ile Ala Arg Leu Thr His Ser Pro Val His Asp Asp Thr Ser Ser
305                 310                 315                 320

Asn His Thr Leu Asp Ser Ser Pro Ala Thr Phe Pro Leu Asn Ser Thr
                325                 330                 335

Leu Tyr Ala Asp Phe Ser His Asp Asn Gly Ile Ile Ser Ile Leu Phe
            340                 345                 350
```

```
Ala Leu Gly Leu Tyr Asn Gly Thr Lys Pro Leu Ser Thr Thr Thr Val
      355                 360                 365

Glu Asn Ile Thr Gln Thr Asp Gly Phe Ser Ser Ala Trp Thr Val Pro
    370                 375                 380

Phe Ala Ser Arg Leu Tyr Val Glu Met Met Gln Cys Gln Ala Glu Gln
385                 390                 395                 400

Glu Pro Leu Val Arg Val Leu Val Asn Asp Arg Val Val Pro Leu His
                405                 410                 415

Gly Cys Pro Val Asp Ala Leu Gly Arg Cys Thr Arg Asp Ser Phe Val
            420                 425                 430

Arg Gly Leu Ser Phe Ala Arg Ser Gly Gly Asp Trp Ala Glu Cys Phe
        435                 440                 445

Ala
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<210> 5
<211> 96
<212> DNA
<213> Daucus carota

<220>
<221> CDS
<222> (1)..(96)

<400> 5

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Met Gly Arg Ile Ala Arg Gly Ser Lys Met Ser Ser Leu Ile Val Ser
  1               5                  10                  15

ttg ctt gta gta ttg gtg tca ctc aat ttg gct tcc gaa acc aca gct   96
Leu Leu Val Val Leu Val Ser Leu Asn Leu Ala Ser Glu Thr Thr Ala
             20                  25                  30
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<210> 6
<211> 32
<212> PRT
<213> Daucus carota

<400> 6

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Leu Leu Val Val Leu Val Ser Leu Asn Leu Ala Ser Glu Thr Thr Ala
             20                  25                  30
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<210> 7
<211> 93
<212> DNA
<213> Lupinus luteus

<220>
<221> CDS
<222> (1)..(93)

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Met Gly Tyr Tyr Ser Ile Tyr Cys Leu Ile Val Leu Val Asn Val Leu
  1               5                  10                  15
```

```
gtg ttt tgc gat gga ggg aag acg agt agt ttt gtt agg gaa tct        93
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            20                  25                  30


<210> 8
<211> 31
<212> PRT
<213> Lupinus ssp.

<400> 8
Met Gly Tyr Tyr Ser Ile Tyr Cys Leu Ile Val Leu Val Asn Val Leu
  1               5                  10                  15

Val Phe Cys Asp Gly Gly Lys Thr Ser Ser Phe Val Arg Glu Ser
            20                  25                  30


<210> 9
<211> 1350
<212> DNA
<213> Aspergillus niger

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<221> CDS
<222> (1)..(1347)

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  1               5                  10                  15

gat cag ggg tat caa tgc ttc tcg gag act tcg cat ctt tgg ggc caa    96
Asp Gln Gly Tyr Gln Cys Phe Ser Glu Thr Ser His Leu Trp Gly Gln
            20                  25                  30

tac gcg ccc ttc ttt tct ctg gca aac aaa tcg gcc atc tcc cct gat   144
Tyr Ala Pro Phe Phe Ser Leu Ala Asn Lys Ser Ala Ile Ser Pro Asp
        35                  40                  45

gtt cct gcc gga tgc cat gtc act ttc gcc cag gtt ctc tcc cgc cat   192
Val Pro Ala Gly Cys His Val Thr Phe Ala Gln Val Leu Ser Arg His
    50                  55                  60

gga gca cgg tat ccg acc gac tcc aag ggc aag aaa tac tcc gct ctc   240
Gly Ala Arg Tyr Pro Thr Asp Ser Lys Gly Lys Lys Tyr Ser Ala Leu
65                  70                  75                  80

atc gag gag atc cag cag aac gcg aca acc ttc gag ggg aaa tat gcc   288
Ile Glu Glu Ile Gln Gln Asn Ala Thr Thr Phe Glu Gly Lys Tyr Ala
                85                  90                  95

ttc ctg aag aca tac aac tac agc ctg ggc gcg gat gat ctg act ccc   336
Phe Leu Lys Thr Tyr Asn Tyr Ser Leu Gly Ala Asp Asp Leu Thr Pro
            100                 105                 110

ttc gga gag cag gag ctg gtc aac tcc ggc gtc aag ttc tac cag cga   384
Phe Gly Glu Gln Glu Leu Val Asn Ser Gly Val Lys Phe Tyr Gln Arg
        115                 120                 125

tac gaa tcg ctc aca aga aac att gtc ccg ttc atc cga tcc tca ggc   432
Tyr Glu Ser Leu Thr Arg Asn Ile Val Pro Phe Ile Arg Ser Ser Gly
    130                 135                 140

tcc aac cgc gtg att gcc tct ggc aat aaa ttc atc gag ggc ttt cag   480
Ser Asn Arg Val Ile Ala Ser Gly Asn Lys Phe Ile Glu Gly Phe Gln
145                 150                 155                 160
```

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agc act aag ctg aag gat cct cgt gcc cag ccc ggc caa tcg tcg gcc   528
Ser Thr Lys Leu Lys Asp Pro Arg Ala Gln Pro Gly Gln Ser Ser Ala
                165                 170                 175

aag atc gac gtg gtc att tca gag gcc agc aca tcc aac aac act ctc   576
Lys Ile Asp Val Val Ile Ser Glu Ala Ser Thr Ser Asn Asn Thr Leu
            180                 185                 190

gat ccg ggc acc tgc acc ggt ttc gaa gat agc gaa ttg gcc gat gac   624
Asp Pro Gly Thr Cys Thr Gly Phe Glu Asp Ser Glu Leu Ala Asp Asp
        195                 200                 205

atc gaa gcc aat ttc acc ggc acg ttc gtc ccc ttc att cgt caa cgt   672
Ile Glu Ala Asn Phe Thr Gly Thr Phe Val Pro Phe Ile Arg Gln Arg
    210                 215                 220

ctg gag aat gac ttg tct ggc gtg tct ctc acg gac aca gaa gtg acc   720
Leu Glu Asn Asp Leu Ser Gly Val Ser Leu Thr Asp Thr Glu Val Thr
225                 230                 235                 240

tac ctc atg gac atg tgc tcc ttg gac acc atc tcc acc agc acc gtc   768
Tyr Leu Met Asp Met Cys Ser Leu Asp Thr Ile Ser Thr Ser Thr Val
                245                 250                 255

gac acc aag ctg tcc ccc ttc tgt gac ctg ttc acc cat gaa gaa tgg   816
Asp Thr Lys Leu Ser Pro Phe Cys Asp Leu Phe Thr His Glu Glu Trp
            260                 265                 270

atc aac tac gac tac ctc cag tcc ccg aac aaa tac tac ggc cat ggc   864
Ile Asn Tyr Asp Tyr Leu Gln Ser Pro Asn Lys Tyr Tyr Gly His Gly
        275                 280                 285

gca ggt aac ccg ctc ggc ccg acc cag ggc gtc ggc tac gct aac gag   912
Ala Gly Asn Pro Leu Gly Pro Thr Gln Gly Val Gly Tyr Ala Asn Glu
    290                 295                 300

ctc atc gcc cgt ctc acc cac tcg cct gtc cac gat gac acc agc tcc   960
Leu Ile Ala Arg Leu Thr His Ser Pro Val His Asp Asp Thr Ser Ser
305                 310                 315                 320

aac cac aca ttg gac tcc aac ccg gct act ttc ccg ctc aac tcc act  1008
Asn His Thr Leu Asp Ser Asn Pro Ala Thr Phe Pro Leu Asn Ser Thr
                325                 330                 335

ctc tat gcg gac ttt tcg cat gat aac ggc atc atc tct atc ctc ttt  1056
Leu Tyr Ala Asp Phe Ser His Asp Asn Gly Ile Ile Ser Ile Leu Phe
            340                 345                 350

gct ttg ggt ctg tac aac ggc acc aag ccg ctg tct tcc acg acc gcg  1104
Ala Leu Gly Leu Tyr Asn Gly Thr Lys Pro Leu Ser Ser Thr Thr Ala
        355                 360                 365

gag aat atc acc cag acc gat ggg ttc tca tct gcc tgg acg gtt cct  1152
Glu Asn Ile Thr Gln Thr Asp Gly Phe Ser Ser Ala Trp Thr Val Pro
    370                 375                 380

ttc gcg tcg cgc atg tac gtc gag atg atg caa tgc cag tcc gag cag  1200
Phe Ala Ser Arg Met Tyr Val Glu Met Met Gln Cys Gln Ser Glu Gln
385                 390                 395                 400

gag cct ttg gtc cgt gtc ttg gtt aat gat cgt gtt gtt ccg ctg cat  1248
Glu Pro Leu Val Arg Val Leu Val Asn Asp Arg Val Val Pro Leu His
                405                 410                 415

ggc tgt ccg gtt gat gct ttg gga aga tgt acg cgg gat agc ttc gtg  1296
Gly Cys Pro Val Asp Ala Leu Gly Arg Cys Thr Arg Asp Ser Phe Val
            420                 425                 430
```

```
aag ggg ttg agc ttt gcc aga tct ggg ggt gat tgg gcg gag tgt ttt   1344
Lys Gly Leu Ser Phe Ala Arg Ser Gly Gly Asp Trp Ala Glu Cys Phe
        435                 440                 445

gct tag                                                          1350
Ala
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<210> 10
<211> 449
<212> PRT
<213> Aspergillus niger

<400> 10

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Met Leu Ala Val Pro Ala Ser Arg Asn Gln Ser Thr Cys Asp Thr Val
  1               5                  10                  15

Asp Gln Gly Tyr Gln Cys Phe Ser Glu Thr Ser His Leu Trp Gly Gln
             20                  25                  30

Tyr Ala Pro Phe Phe Ser Leu Ala Asn Lys Ser Ala Ile Ser Pro Asp
         35                  40                  45

Val Pro Ala Gly Cys His Val Thr Phe Ala Gln Val Leu Ser Arg His
     50                  55                  60

Gly Ala Arg Tyr Pro Thr Asp Ser Lys Gly Lys Lys Tyr Ser Ala Leu
 65                  70                  75                  80

Ile Glu Glu Ile Gln Gln Asn Ala Thr Thr Phe Glu Gly Lys Tyr Ala
                 85                  90                  95

Phe Leu Lys Thr Tyr Asn Tyr Ser Leu Gly Ala Asp Asp Leu Thr Pro
            100                 105                 110

Phe Gly Glu Gln Glu Leu Val Asn Ser Gly Val Lys Phe Tyr Gln Arg
        115                 120                 125

Tyr Glu Ser Leu Thr Arg Asn Ile Val Pro Phe Ile Arg Ser Ser Gly
    130                 135                 140

Ser Asn Arg Val Ile Ala Ser Gly Asn Lys Phe Ile Glu Gly Phe Gln
145                 150                 155                 160

Ser Thr Lys Leu Lys Asp Pro Arg Ala Gln Pro Gly Gln Ser Ser Ala
                165                 170                 175

Lys Ile Asp Val Val Ile Ser Glu Ala Ser Thr Ser Asn Asn Thr Leu
            180                 185                 190

Asp Pro Gly Thr Cys Thr Gly Phe Glu Asp Ser Glu Leu Ala Asp Asp
        195                 200                 205

Ile Glu Ala Asn Phe Thr Gly Thr Phe Val Pro Phe Ile Arg Gln Arg
    210                 215                 220

Leu Glu Asn Asp Leu Ser Gly Val Ser Leu Thr Asp Thr Glu Val Thr
225                 230                 235                 240

Tyr Leu Met Asp Met Cys Ser Leu Asp Thr Ile Ser Thr Ser Thr Val
                245                 250                 255

Asp Thr Lys Leu Ser Pro Phe Cys Asp Leu Phe Thr His Glu Glu Trp
            260                 265                 270

Ile Asn Tyr Asp Tyr Leu Gln Ser Pro Asn Lys Tyr Tyr Gly His Gly
        275                 280                 285
```

```
Ala Gly Asn Pro Leu Gly Pro Thr Gln Gly Val Gly Tyr Ala Asn Glu
    290                 295                 300

Leu Ile Ala Arg Leu Thr His Ser Pro Val His Asp Asp Thr Ser Ser
305                 310                 315                 320

Asn His Thr Leu Asp Ser Asn Pro Ala Thr Phe Pro Leu Asn Ser Thr
                325                 330                 335

Leu Tyr Ala Asp Phe Ser His Asp Asn Gly Ile Ile Ser Ile Leu Phe
            340                 345                 350

Ala Leu Gly Leu Tyr Asn Gly Thr Lys Pro Leu Ser Ser Thr Thr Ala
        355                 360                 365

Glu Asn Ile Thr Gln Thr Asp Gly Phe Ser Ser Ala Trp Thr Val Pro
    370                 375                 380

Phe Ala Ser Arg Met Tyr Val Glu Met Met Gln Cys Gln Ser Glu Gln
385                 390                 395                 400

Glu Pro Leu Val Arg Val Leu Val Asn Asp Arg Val Val Pro Leu His
                405                 410                 415

Gly Cys Pro Val Asp Ala Leu Gly Arg Cys Thr Arg Asp Ser Phe Val
            420                 425                 430

Lys Gly Leu Ser Phe Ala Arg Ser Gly Gly Asp Trp Ala Glu Cys Phe
        435                 440                 445

Ala
```

DATED this TWENTY FOURTH day of SEPTEMBER, 1999

Commonwealth Scientific and Industrial Research Organisation

AND Australian Wool Research and Promotion Organisation

By DAVIES COLLISON CAVE

Patent Attorneys for the Applicants

ClustalW Formatted Alignments

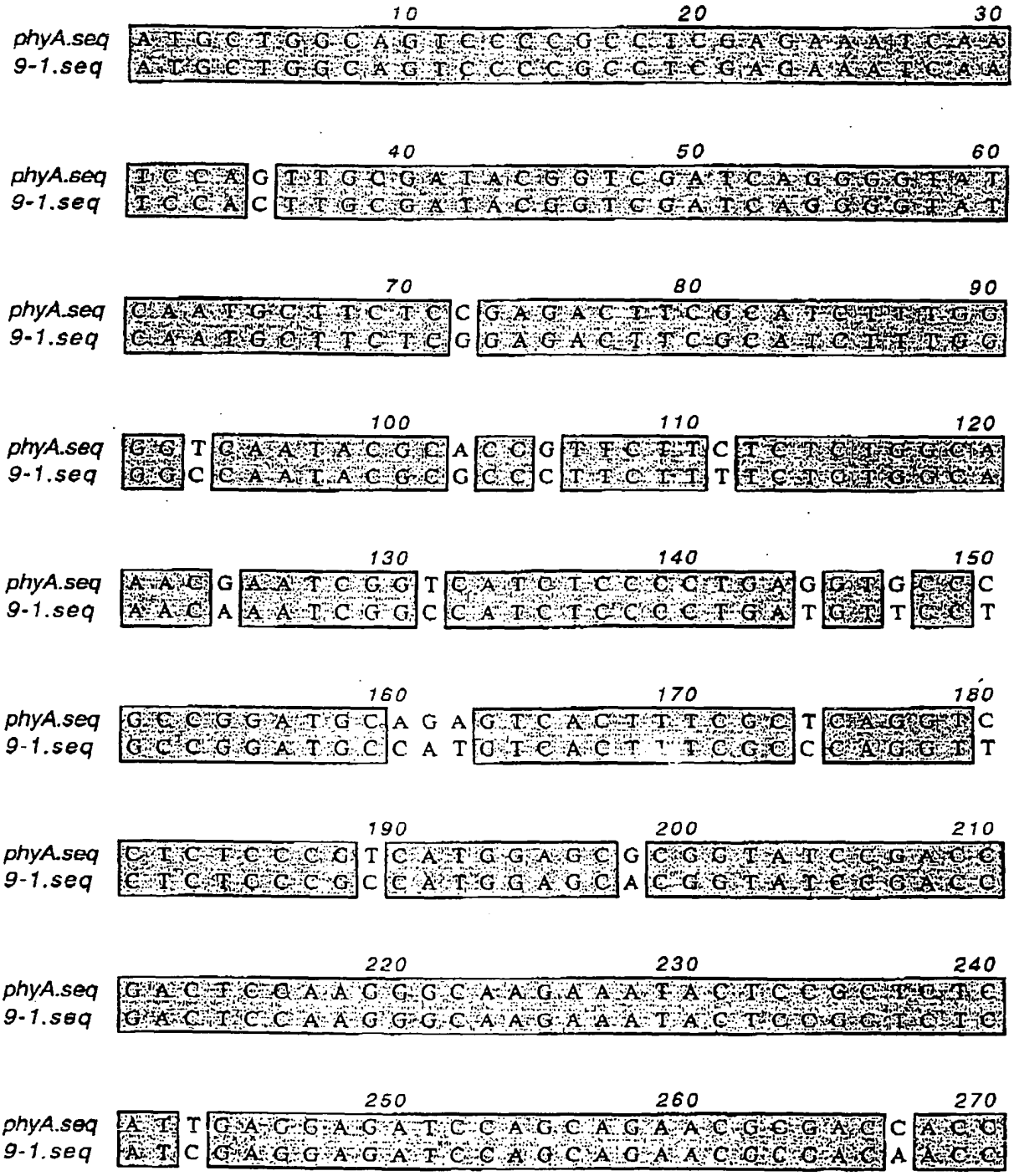

FIGURE 1 - 1

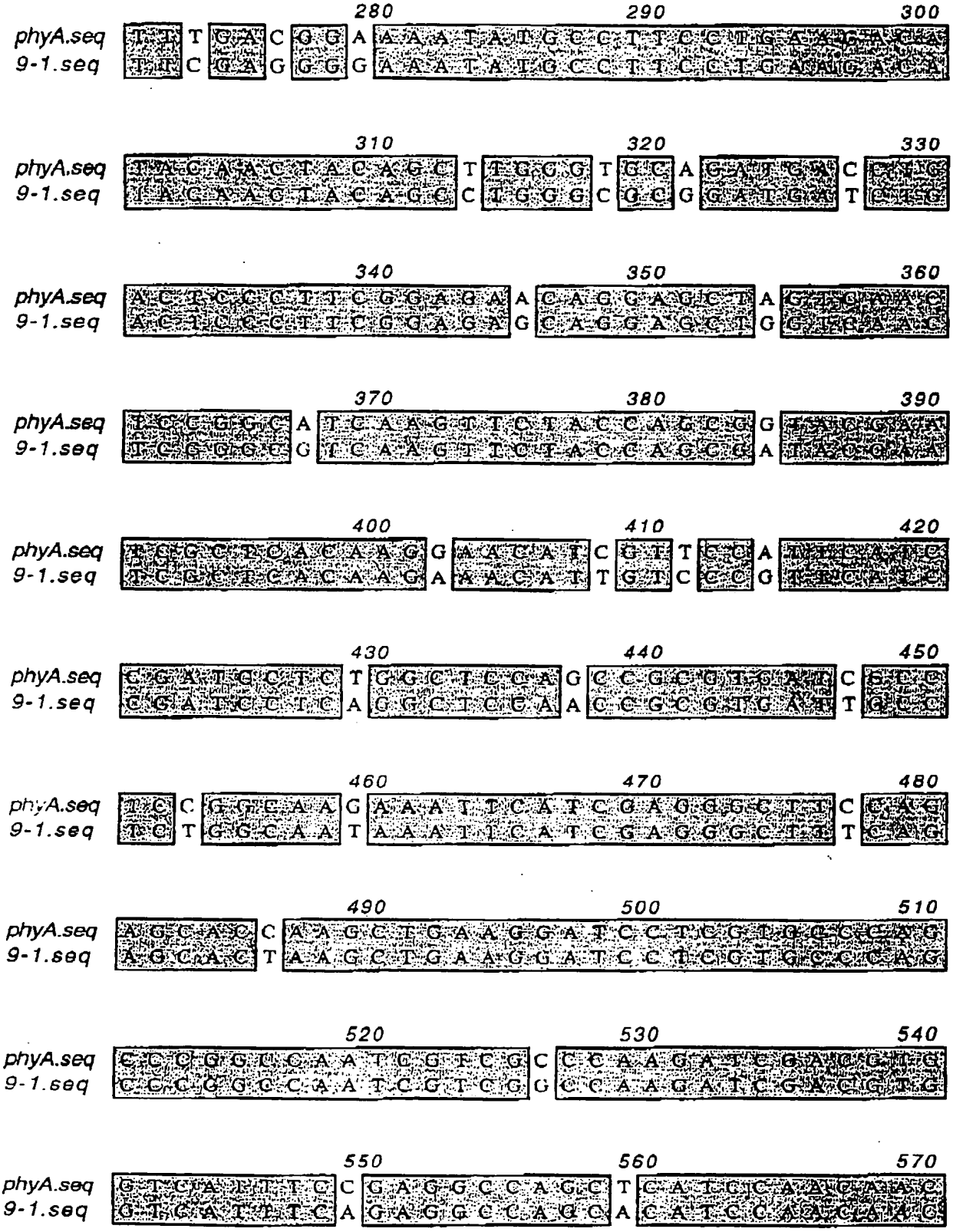
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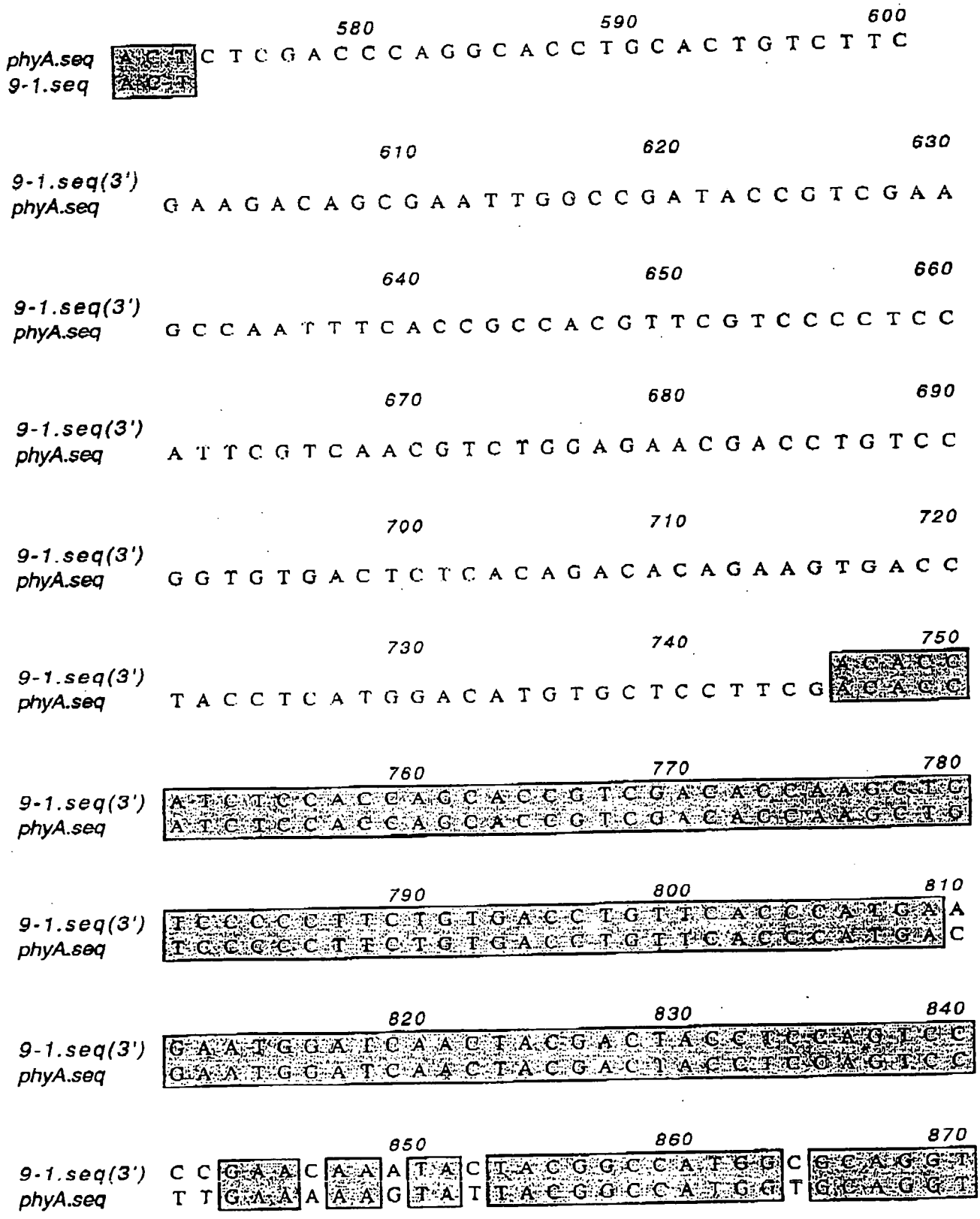
FIGURE 1 - 2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phyA.seq

FIGURE 1 - 3

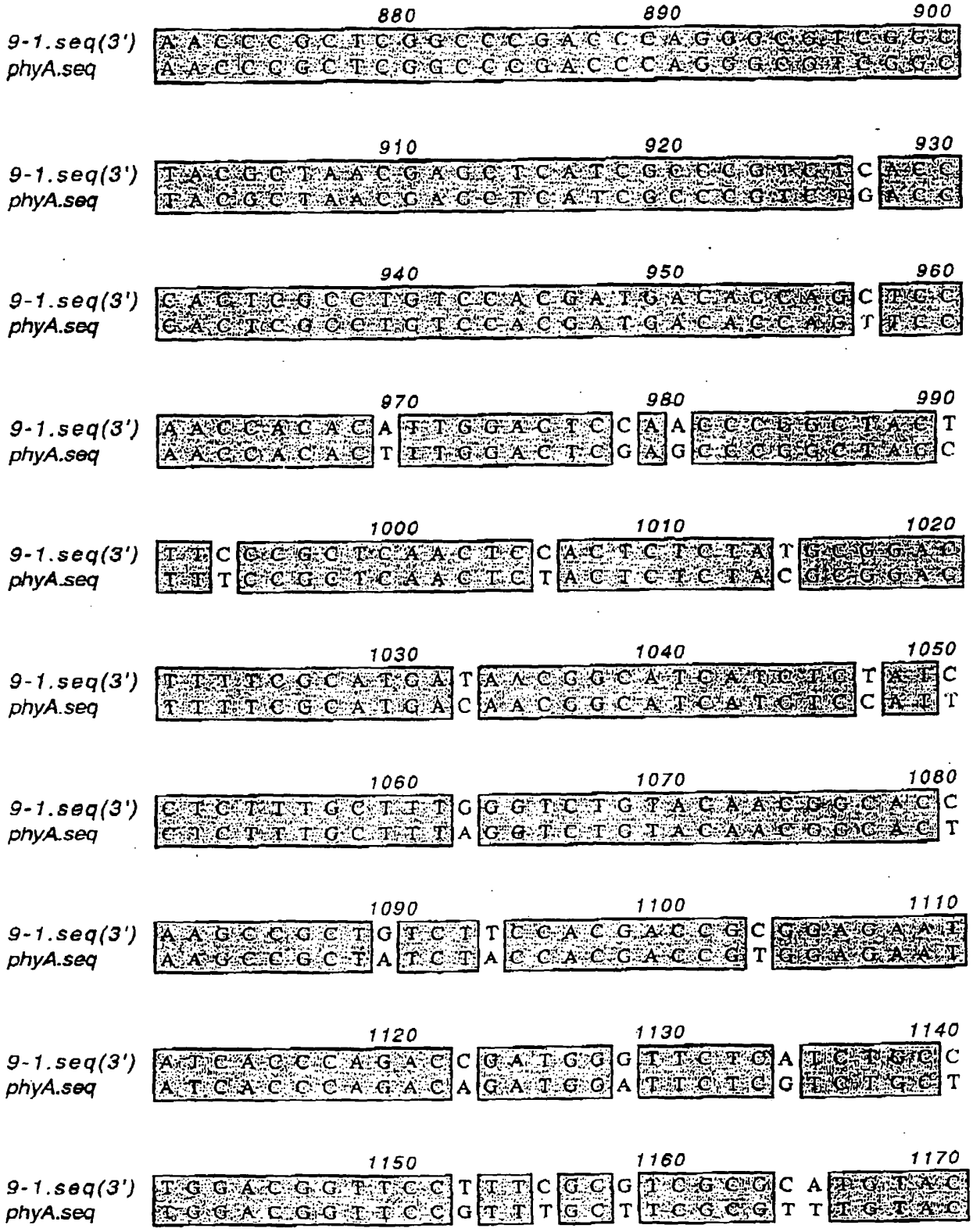

880
890
900
9-1.seq(3')
phyA.seq
910
920
930
940
950
960
970
980
990
1000
1010
1020
1030
1040
1050
1060
1070
1080
1090
1100
1110
1120
1130
1140
1150
1160
1170

FIGURE 1 - 4

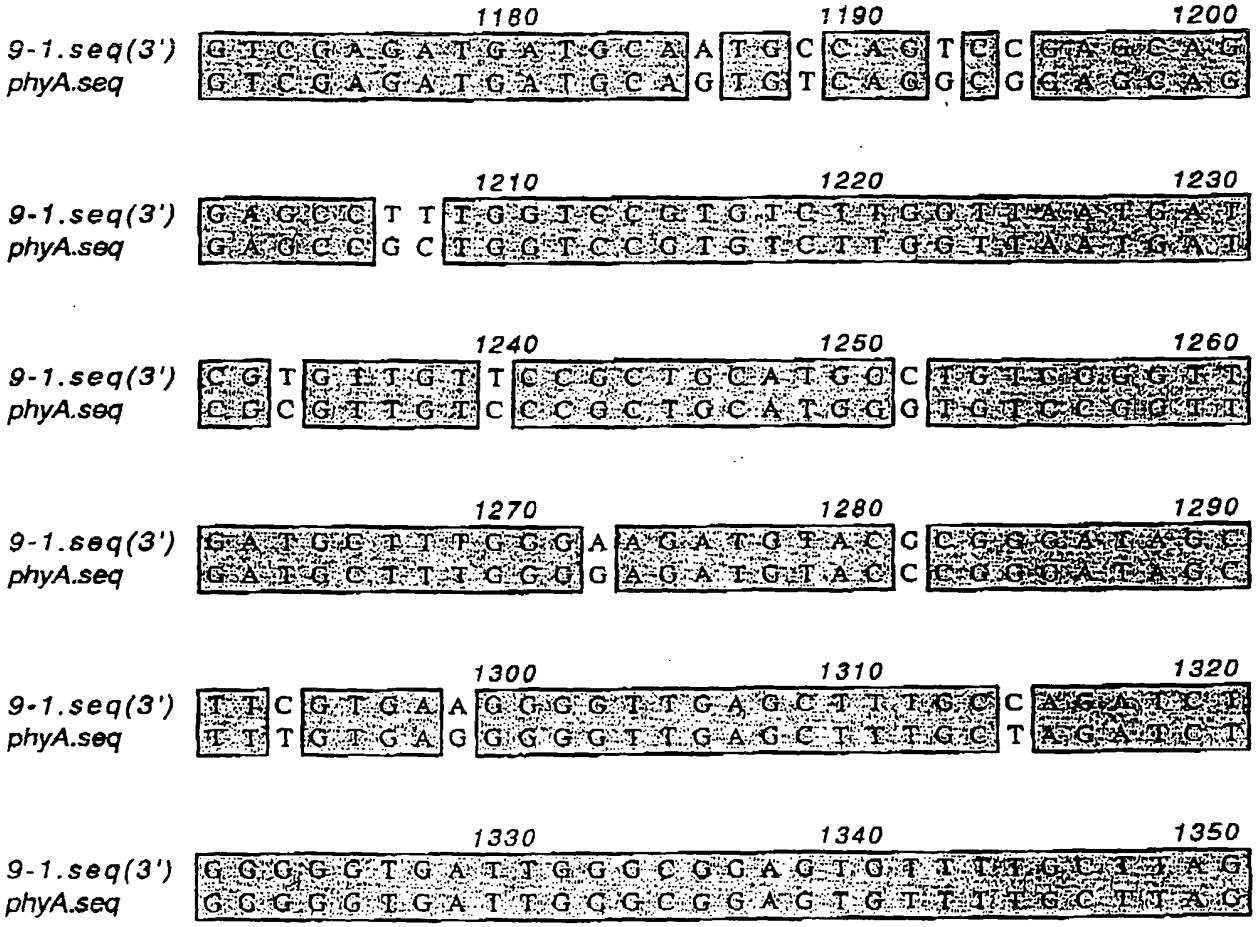

1180
1190
1200
9-1.seq(3')
phyA.seq
GTCGAGATGATGCAATGCCAGTCCGTAGCAAG
GTCGAGATGATGCAGTGTCAGGCGGAGCAAG
1210
1220
1230
9-1.seq(3')
phyA.seq
GAGCCTTTGGTCCGTGTCTTGGTTTAATGAT
GAGCCGCTGGTCCGTGTCTTGGTTAATGAT
1240
1250
1260
9-1.seq(3')
phyA.seq
CGTGTTGTTCCGCTGCATGCCTGTCGGGTT
CGCGTTGTCCCGCTGCATGGGTGTCCGGTT
1270
1280
1290
9-1.seq(3')
phyA.seq
GATGCTTTGGGAAGATGTACCCGGGATAGC
GATGCTTTGGGGAGATGTACCCGGGATAGC
1300
1310
1320
9-1.seq(3')
phyA.seq
TTCGTGAAGGGGTTGAGCTTTGCCAGATCT
TTTGTGAGGGGGTTGAGCTTTGCTAGATCT
1330
1340
1350
9-1.seq(3')
phyA.seq
GGGGGTGATTGGGCGGAGTGTTTTGCTTAG
GGGGGTGATTGGCGGGAGTGTTTTGCTTAG

FIGURE 1 - 5

ClustalW Formatted Alignments

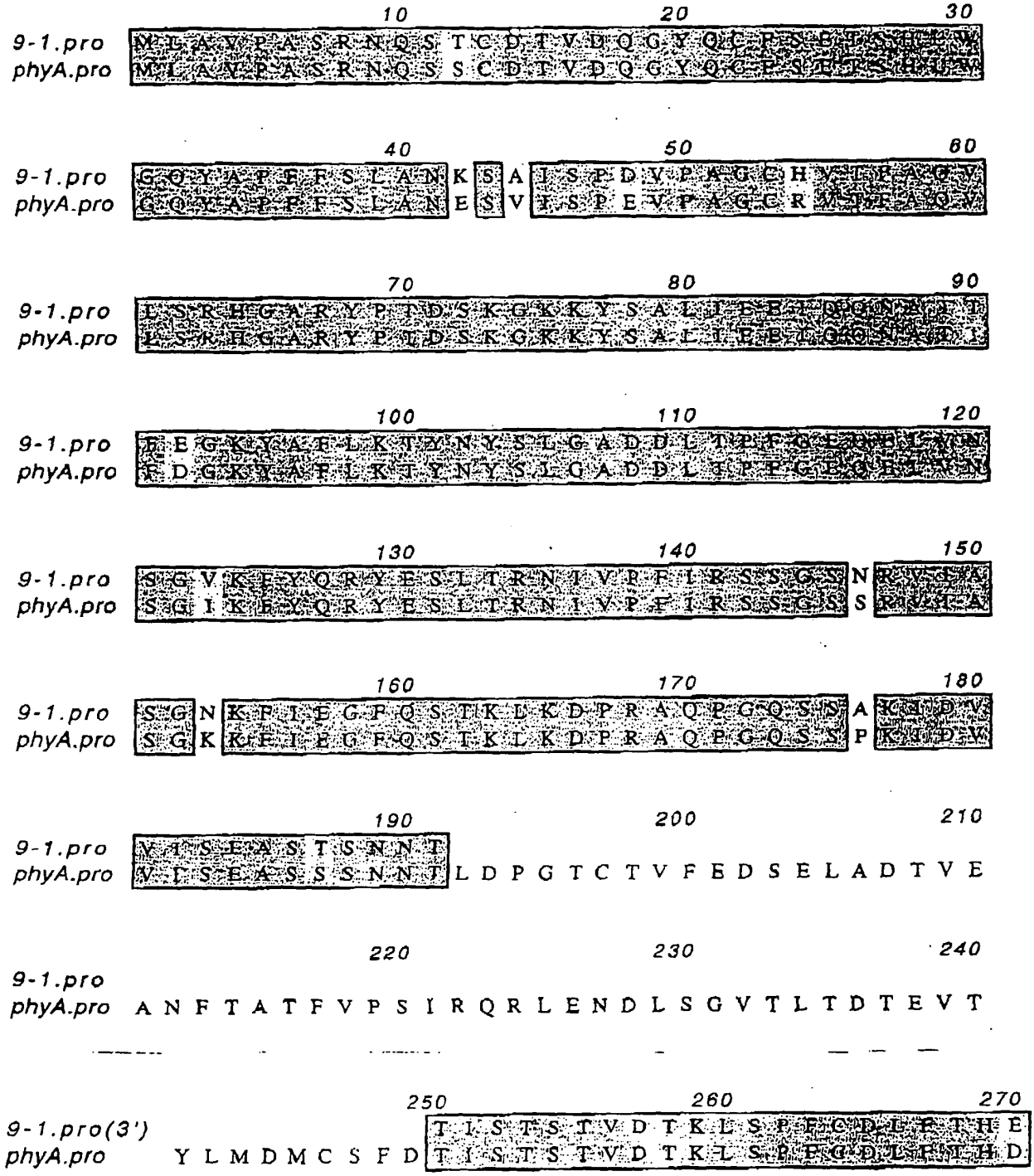


FIGURE 2 - 1

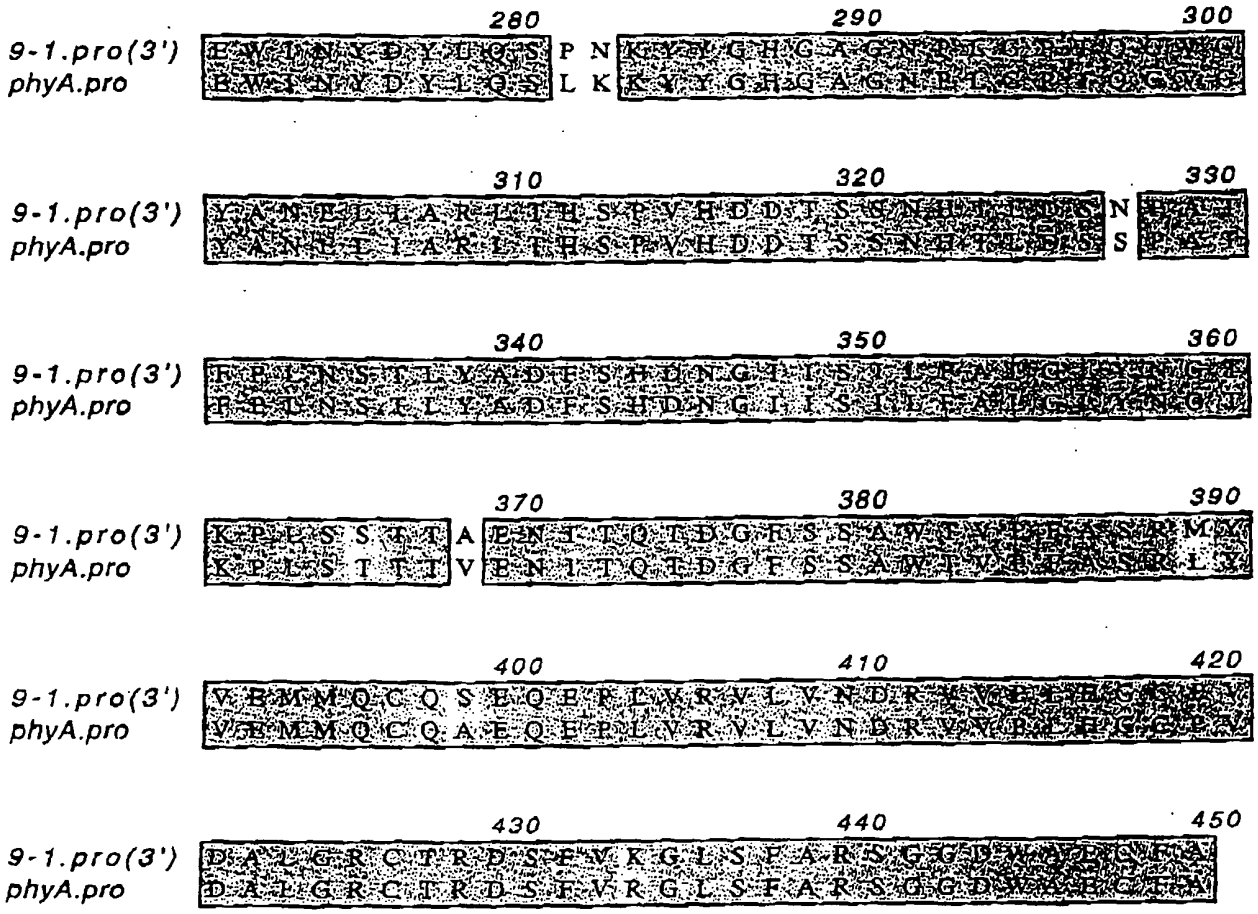

280
290
300
9-1.pro(3')
phyA.pro
310
320
330
9-1.pro(3')
phyA.pro
340
350
360
9-1.pro(3')
phyA.pro
370
380
390
9-1.pro(3')
phyA.pro
400
410
420
9-1.pro(3')
phyA.pro
430
440
450
9-1.pro(3')
phyA.pro

FIGURE 2 - 2

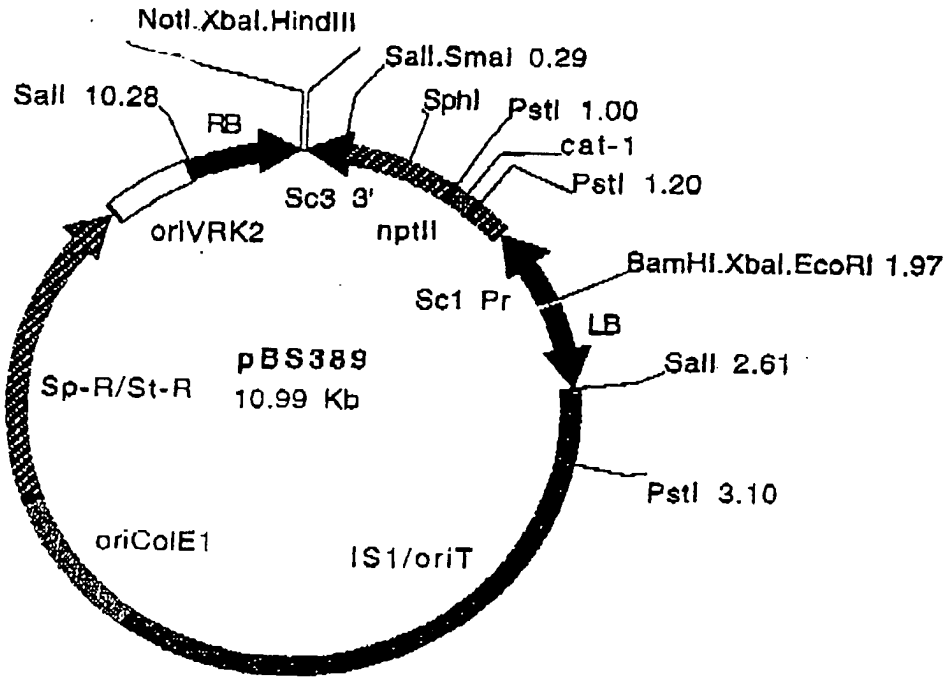

NotI.XbaI.HindIII
SalI.SmaI 0.29
SalI 10.28
RB
SphI
PstI 1.00
cat-1
PstI 1.20
Sc3 3'
oriVRK2
nptII
BamHI.XbaI.EcoRI 1.97
Sc1 Pr
LB
pBS389
10.99 Kb
SalI 2.61
Sp-R/St-R
PstI 3.10
oriColE1
IS1/oriT

FIGURE 3

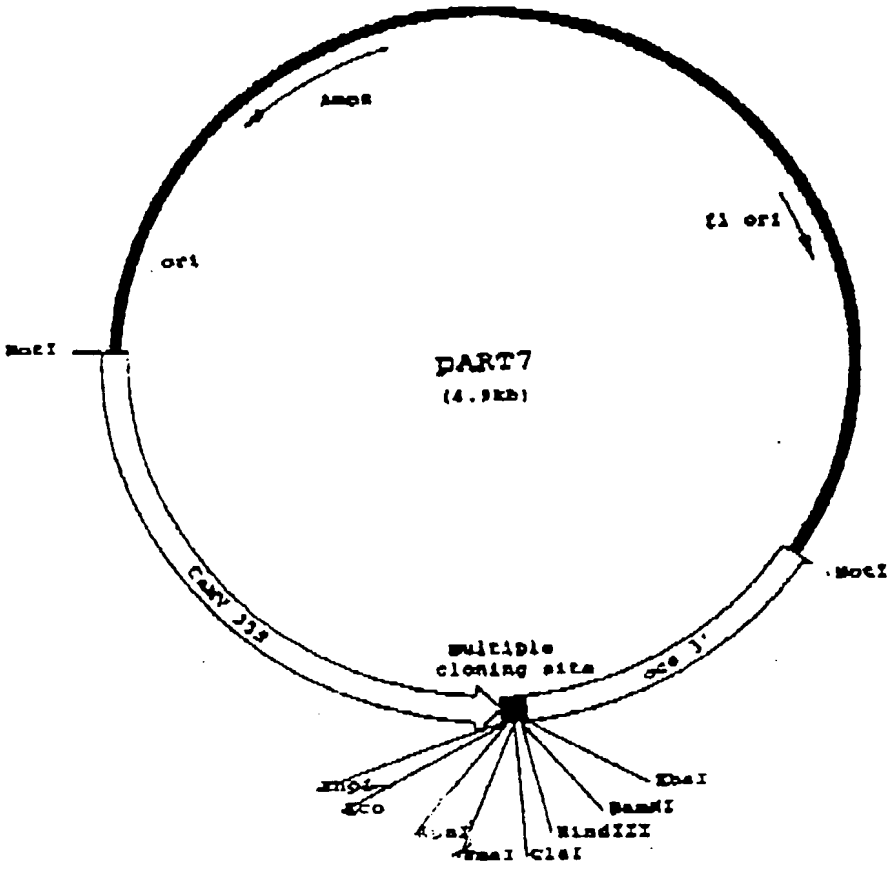

Amp^R
f1 ori
ori
NotI
pART7
(4.9Kb)
NotI
CMV 3'
multiple
cloning site
gc 3'
XbaI
BamHI
HindIII
ClaI

FIGURE 4

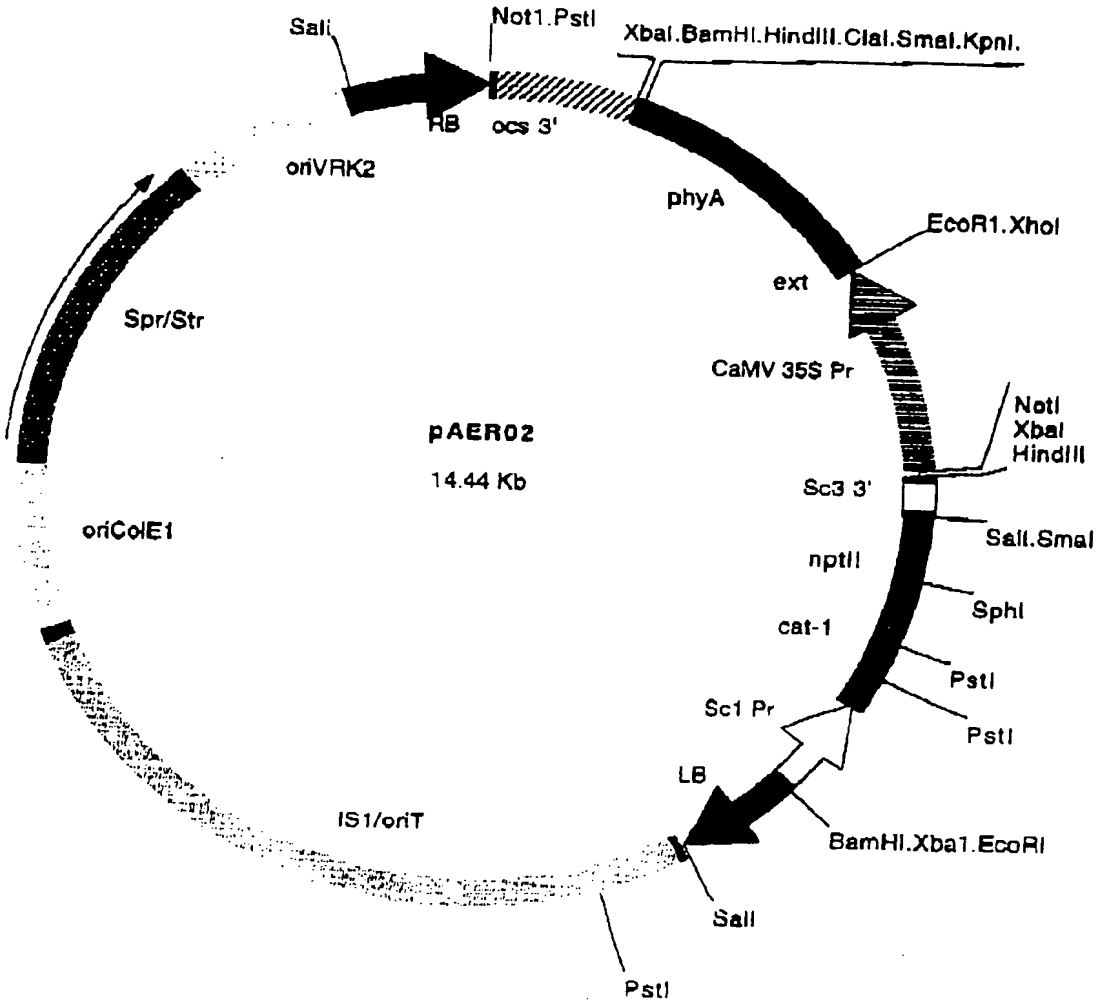

SalI
Not1.PstI
XbaI.BamHI.HindIII.ClaI.SmaI.KpnI.
RB
ocs 3'
oriVRK2
phyA
EcoR1.XhoI
ext
Spr/Str
CaMV 35S Pr
NotI
XbaI
HindIII
pAER02
14.44 Kb
Sc3 3'
oriColE1
SalI.SmaI
nptII
SphI
cat-1
PstI
Sc1 Pr
PstI
LB
IS1/oriT
BamHI.Xba1.EcoRI
SalI
PstI

FIGURE 5

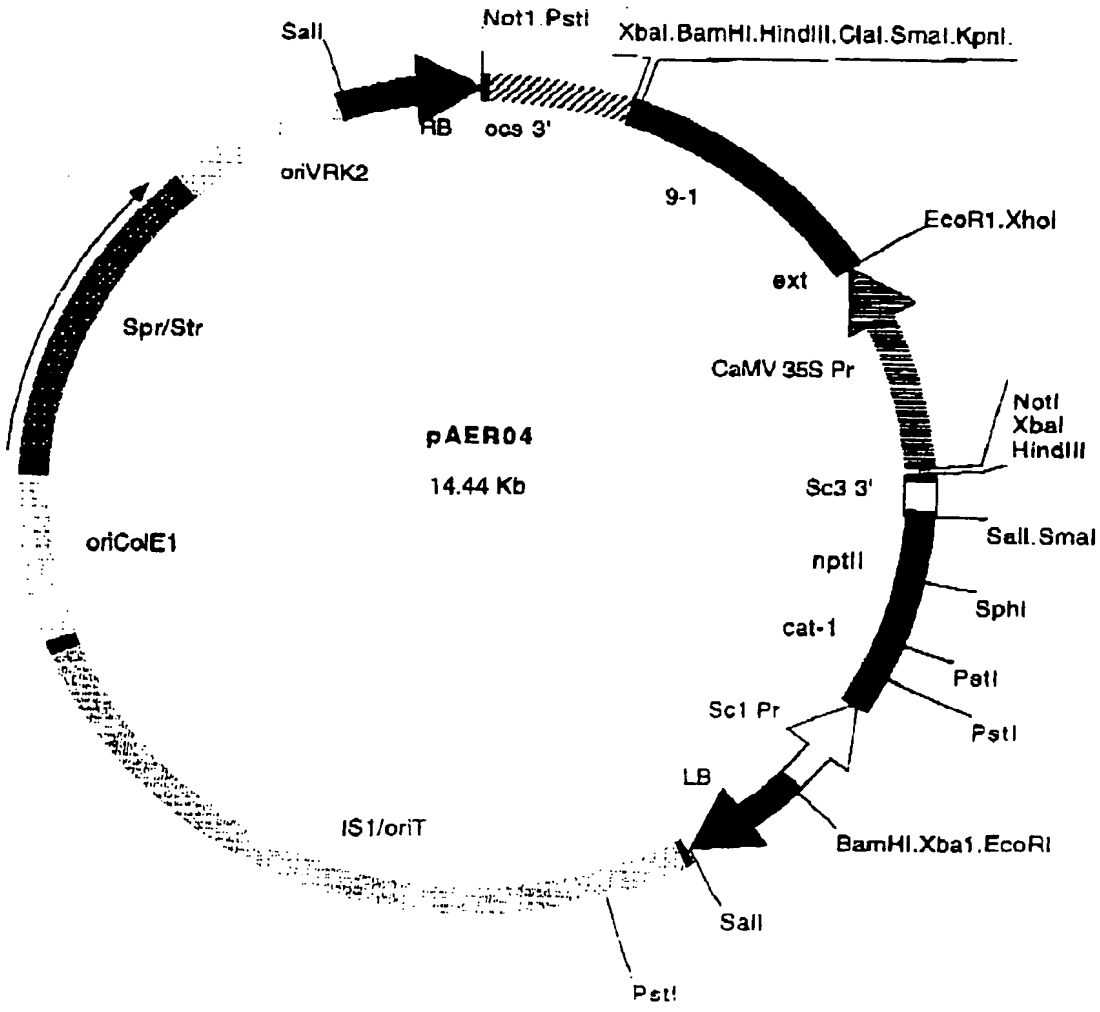

SalI
Not1 PstI
XbaI.BamHI.HindIII.ClaI.SmaI.KpnI.
RB
ocs 3'
oriVRK2
9-1
EcoR1.XhoI
ext
Spr/Str
CaMV 35S Pr
NotI
XbaI
HindIII
pAER04
14.44 Kb
Sc3 3'
SalI.SmaI
oriColE1
nptII
SphI
cat-1
PstI
Sc1 Pr
PstI
LB
IS1/oriT
BamHI.Xba1.EcoRI
SalI
PstI

FIGURE 6

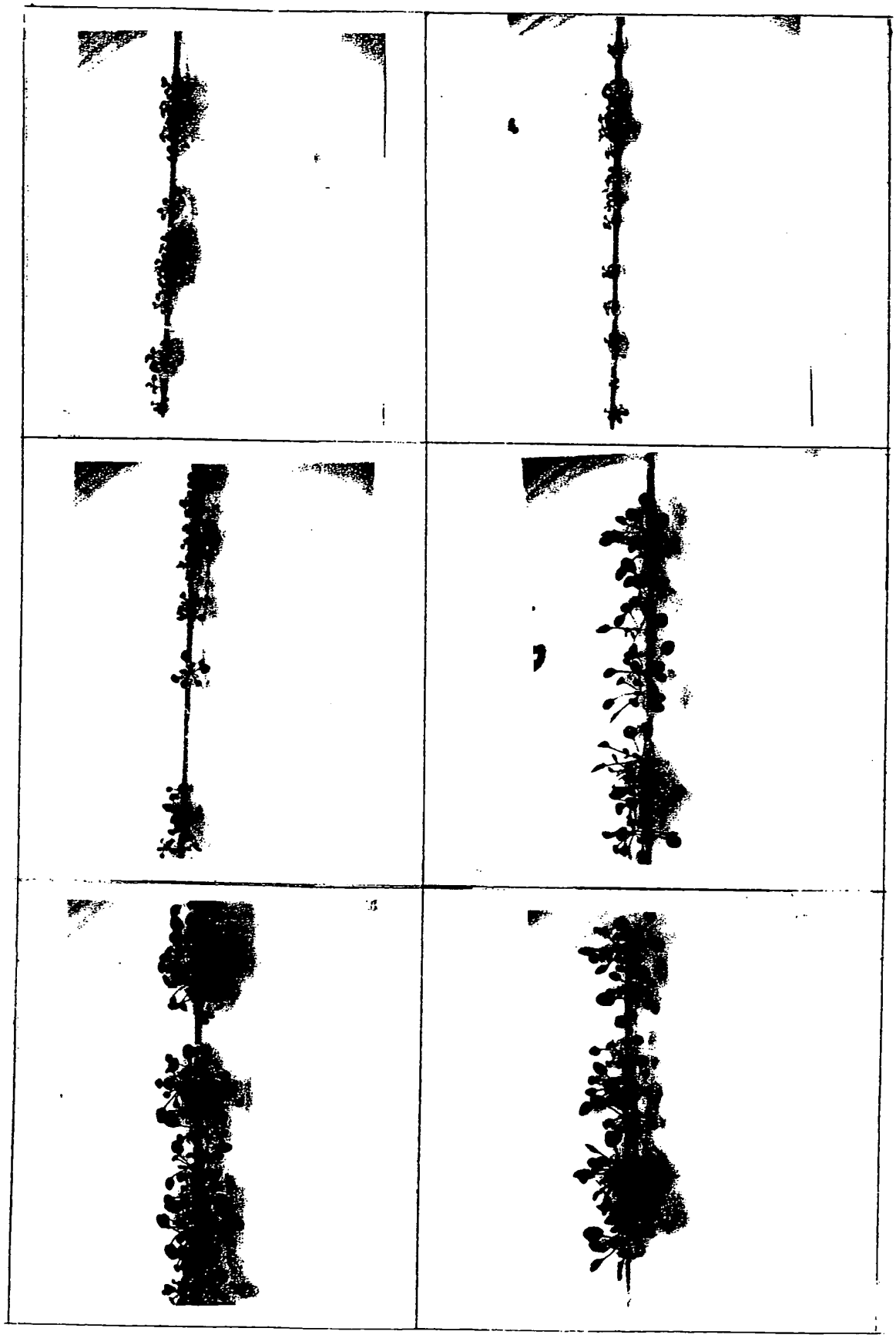


FIGURE 7

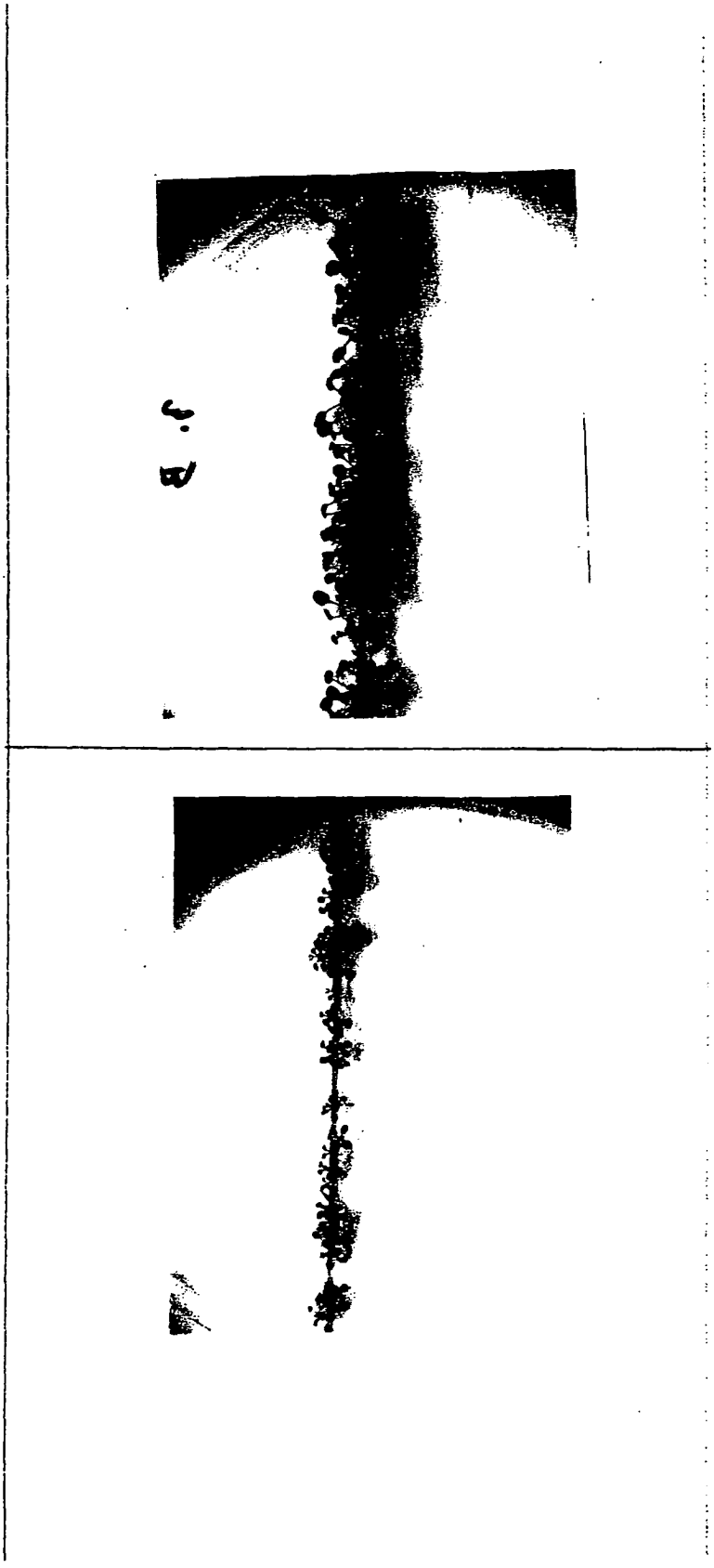


FIGURE 8

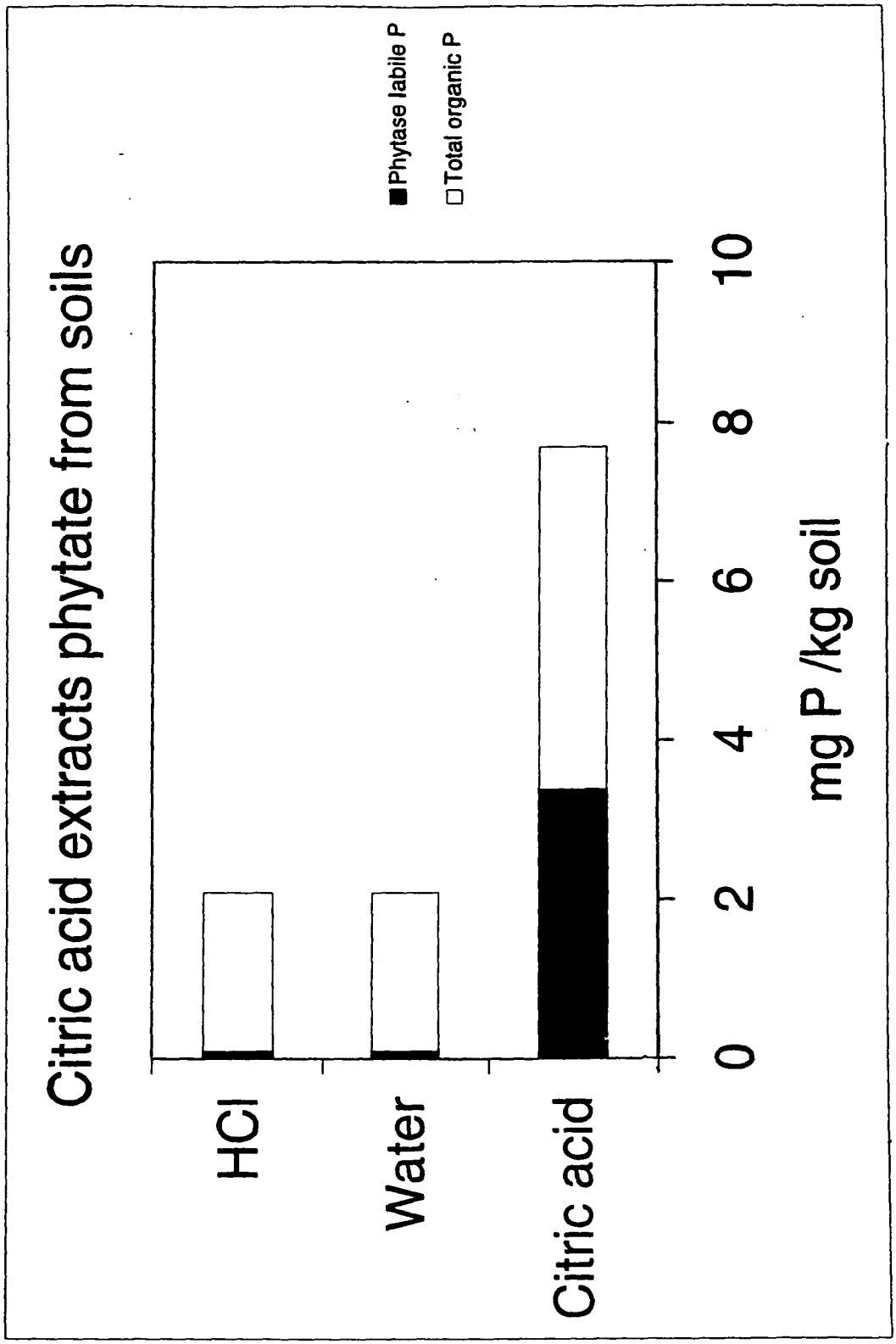

Citric acid extracts phytate from soils
Phytase labile P
Total organic P
HCl
Water
Citric acid
0
2
4
6
8
10
mg P /kg soil

FIGURE 9

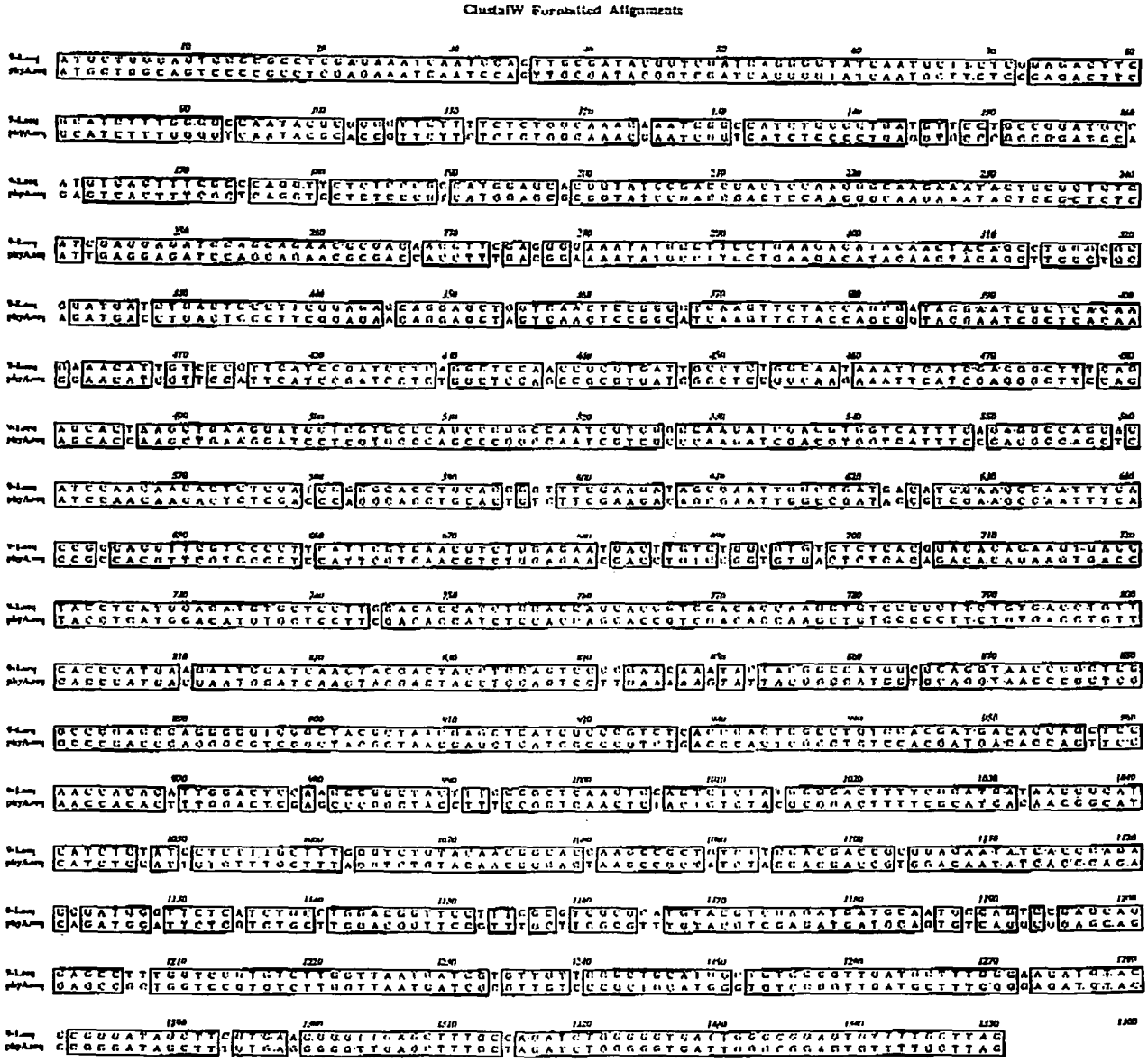


FIGURE 10

ClustalW Formatted Alignments

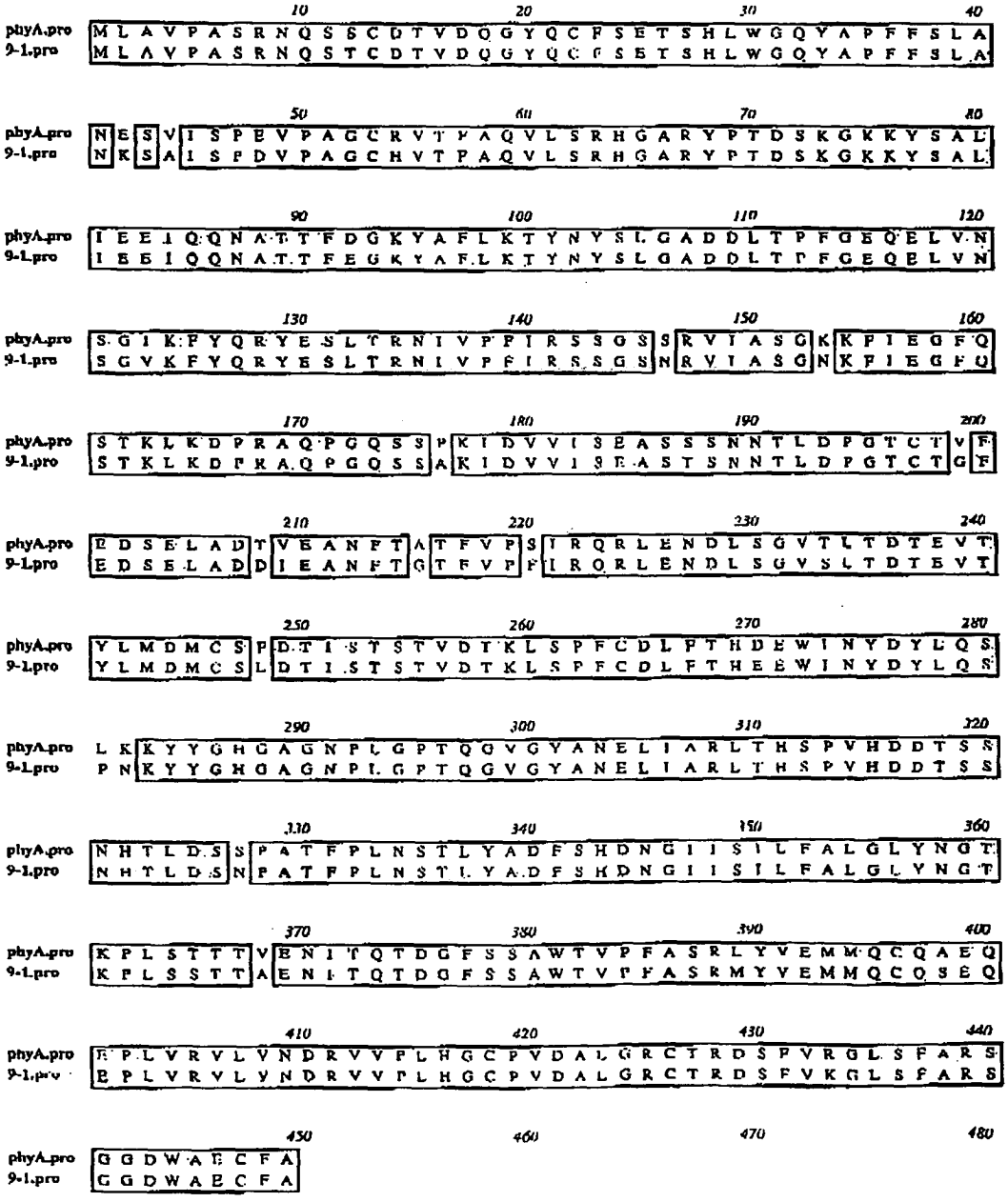


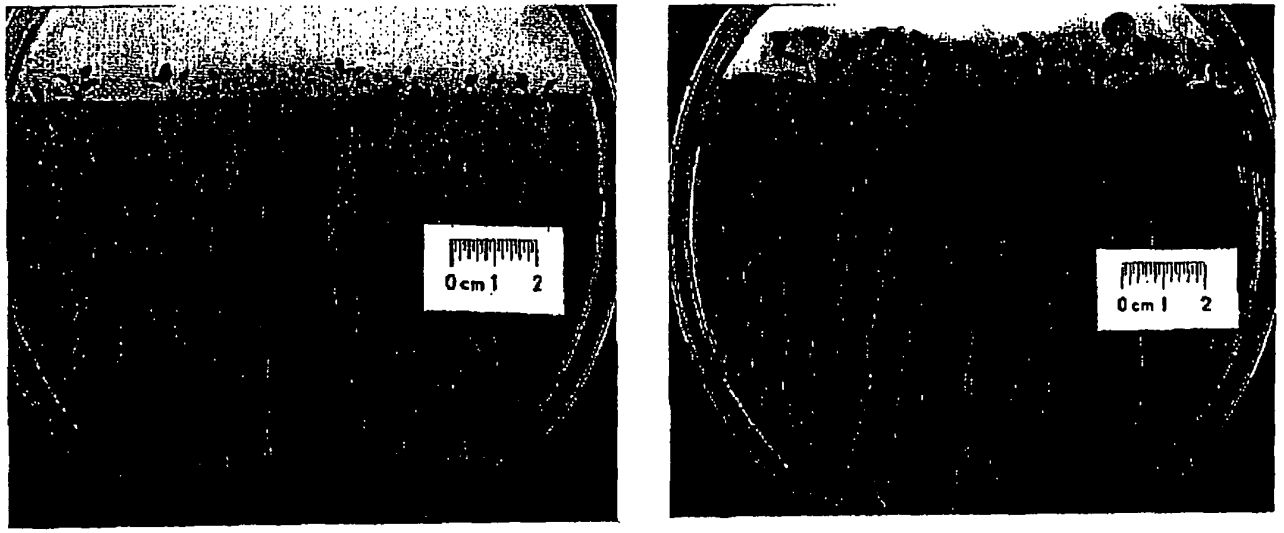
FIGURE 11

CSIRO
PLANT
INDUSTRY
- Phosphorus (No P)
+ Phosphorus (Pi)
Control plants: 40 days growth

FIGURE 12

CSIRO
PLANT
INDUSTRY

Phosphorus as Phytate



Control plants

+ Phytase gene

FIGURE 13